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Effect of Iron Binding on the Ability of Crocidolite to Cause DNA Single-Strand Breaks

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EFFECT OF IRON BINDING ON THE ABILITY OF CROCIDOLITE TO CAUSE DNA SINGLE-STRAND BREAKS

by

Jeanne Ann Hardy

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Genius may conceive, but patient labor must consummate.

Horace Mann

To my parents

Alan and Diana

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Jeanne A. Hardy

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LIST OF ABBREVIATIONS

8-OHdG	8-hydroxy-2'-deoxyguanosine
ddH_2O	distilled-deionized water
desferrioxamine B	N-[5-[3-[5-(X)hydroxy-acetamide) pentyl]carbamoyl] propionohydroxamic acid monomethaesulfonate
DF crocidolite	Crocidolite treated with desferrioxamine B for 90 days to remove iron.
DMEM	Dulbecco's Modified Eagle Medium
DMPO	5,5'-dimethyl-1-pyrroline-N-oxide
DNA SSB	DNA single-strand break
DPM	Disintintegrations per minute
EDTA	ethylenediaminetetraacetate
EPR	electron paramagnetic resonance spectroscopy
F-12	Ham's F-12 tissue culture medium prepared free of iron salts
Fe F-12	Ham's F-12 tissue culture medium containing 6 μM $FeSO_4$
FeIMDM	Iscove's Modified Dulbecco's Medium with 3 μM Fe added
FeMEM	Minimum Essential Medium with 3μM Fe added
ferrozine	3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p-sulphonic acid
ICP	inductively coupled plasma emission spectrometer
IMDM	Iscove's Modified Dulbecco's Medium
MEM	Minimum Essential Medium
NTA	nitrilotriacetate

PBS	phosphate buffered saline
SiCW	silicon carbide whiskers
SSB	single-strand break

ABSTRACT

Effect of Iron Loading on the Ability of
Crocidolite to Induce DNA Single-Strand Breaks

by

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Fibrous carcinogens, such as crocidolite asbestos, are known to catalyze many of the same reactions as iron, namely O_2 consumption, generation of reduced oxygen species, and damage to DNA, such as strand breaks, and modifications of bases. Upon inhalation, fibers are also known to become coated with an iron-rich material. The mechanism by which this iron is bound to fibers in the lung is not known, and the effect of this additional iron on the reactivity of the fibers is also not well understood. The studies described here were undertaken to elucidate the abilities of crocidolite asbestos, in its native, soaked, and iron-depleted forms, as well as three varieties of silicon carbide whiskers, to acquire reactive iron on their surfaces. The aim has been to quantitate the amount of iron that can bind in short periods of time, and to measure any changes in biochemical reactivity toward DNA following

binding of iron. All forms of the naturally occurring mineral fiber crocidolite, and the man-made mineral fibers (silicon carbide whiskers), were capable of acquiring iron, to varying degrees. Native crocidolite was able to bind up to 57 nmol Fe^{+2}/mg crocidolite in one hour, while the iron-depleted form was capable of binding only 5.5 nmol Fe^{+2}/mg crocidolite, and the three varieties of silicon carbide whiskers bound from 2.9 to 29.0 nmol Fe^{+2}/mg in the same time period. Following iron binding, the fibers were more capable of forming DNA single-strand breaks. The increase in the ability of the fibers to cause DNA strand breaks was greatest with the silicon carbide whiskers, less with iron-depleted crocidolite, and the least with native crocidolite, which is likely because of the inherently high iron content of native crocidolite. Other investigation attempted to determine whether iron could be bound from more complex, physiologically relevant iron-containing solutions where potential iron chelators are abundant. Iron appeared to be acquired even from such complex mixtures as tissue culture media. Following incubation in media, the fibers were more active in catalyzing the formation of DNA strand breaks. An interesting correlation was noted between the abilities of the fibers to cause DNA strand breaks after incubation in tissue culture media and the cytotoxicity of crocidolite to A549 cells grown in the same media. (186 pages)

CHAPTER 1

IRON IN ASBESTOS CHEMISTRY AND CARCINOGENICITY[1]

I. Introduction

Asbestos has been widely used for the manufacture of many products because of its remarkable durability, tensile strength, flame retarding capacity, and slow dissolution properties.[1] In the late 1950s, reports of lung cancer in asbestos miners and millers appeared. Since that time it has become well established that asbestos causes pulmonary interstitial fibrosis; mesothelioma of the pleura, pericardium, and peritoneum;[2] and carcinoma of the lungs, esophagus, and stomach.[3-7] Because of these documented carcinogenic effects in humans, the use of asbestos for most applications was banned in the United States almost two decades ago, eliminating exposure in the workplace. It has been estimated that over 27 million people in the United States alone have had occupational exposure to asbestos between 1940 and 1979, of which 9,000 die from asbestos-related cancers each year.[8] Since these cancers do not appear until 20 or more years after the first exposure to asbestos, the health effects of these occupational exposures may be seen for some years to come. Concern about exposure to ambient levels of asbestos in structures built before the ban and in the air and water surrounding mining operations

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[1]Coauthored by Jeanne A. Hardy and Dr. Ann E. Aust

continues. However, the health consequences of these lower-level exposures are the subject of much debate.

Since the first reports of lung tumors in asbestos miners, there has been an intensive research effort to understand the mechanism by which asbestos causes cancer. Although fiber dimension and durability have been shown to be important determinants of the carcinogenic potential, the molecular mechanism by which asbestos causes cancer remains to be elucidated. Thus, the manufacture of replacement materials may produce fibers which are as carcinogenic as, or more carcinogenic than asbestos itself.

Asbestos is a commercial term used for a group of crystalline silicates. There are two major subdivisions of asbestos minerals, amphibole and serpentine. Crocidolite and amosite, members of the amphibole family, contain high levels of iron[9] and are very carcinogenic. Chrysotile contains little or no iron and is a member of the serpentine, meaning lizard-like, asbestos mineral family.[9] Chrysotile is much less carcinogenic in laboratory animals than the amphiboles. Whether or not it is carcinogenic in man is the subject of debate.[10]

Erionite is a naturally occurring member of the zeolite family of mineral fibers and is not classified as an asbestos mineral. Erionite is not used commercially. It was first shown to be carcinogenic when Turkish villagers, living in caves carved from an outcropping of the mineral,

showed a one-hundred fold increased incidence of mesothelioma.[11,12] Erionite normally contains little or no iron, but is more carcinogenic than any form of asbestos both in man[11,13] and in laboratory animals.[14-21] The mechanism by which this mineral causes cancer is also not understood. Erionite, like other zeolites, has the ability to undergo cation exchange to acquire metals. This cation exchange capability is why zeolites are used in industry as solid catalysts for many types of reactions, including oxidation-reduction reactions. The unusual chemical properties of erionite, a highly carcinogenic mineral, led to its inclusion in this review for comparison with the carcinogenic forms of asbestos.

Evidence is accumulating to suggest that chemical reactions catalyzed by asbestos and other mineral fibers may be responsible for their pathological effects. Asbestos is known to catalyze many of the same reactions that iron does, such as lipid peroxidation, DNA strand breaks, formation of oxidized nucleotide bases, and oxidative damage to protein. This review will address the role of iron in the chemical and physiological reactions attributed to asbestos and will enumerate several lines of evidence which point to the involvement of iron from asbestos in the causation of disease.

The intent of this thesis has been to investigate the ability of iron to bind to crocidolite, crocidolite which

had been treated with desferrioxamine B (DF crocidolite), and three varieties of silicon carbide whiskers, from aqueous solution. Later sections in this chapter will make clear why these studies are warranted, and the potential changes in physiological interaction which may occur as a result of intracellular iron binding. This chapter comprises a comprehensive review of the role of iron in the chemistry and carcinogenicity of asbestos fibers. Chapter 2 reports the abilities of native crocidolite, soaked crocidolite, DF crocidolite, and silicon carbide to bind iron from aqueous solution. Chapter 3 addresses the ability of both crocidolite and DF crocidolite to acquire iron from tissue culture media and the effect of incubation in these media on the *in vitro* reactivity of the fibers.

This chapter was written as a review article in the style of the journal in which it will be published. Chapters 2 and 3 have been written in the format of the journal in which they may be published. Additional information was included for clarity and completeness which was not included in the submitted articles. Each chapter contains its own abstract, introduction, materials and methods, results, discussion, and list of references.

II. Structure of Asbestos and Erionite

Figure 1 shows the structures of crocidolite, amosite, chrysotile, and erionite. The amphibole minerals, crocidolite and amosite, are composed of stacked, infinite,

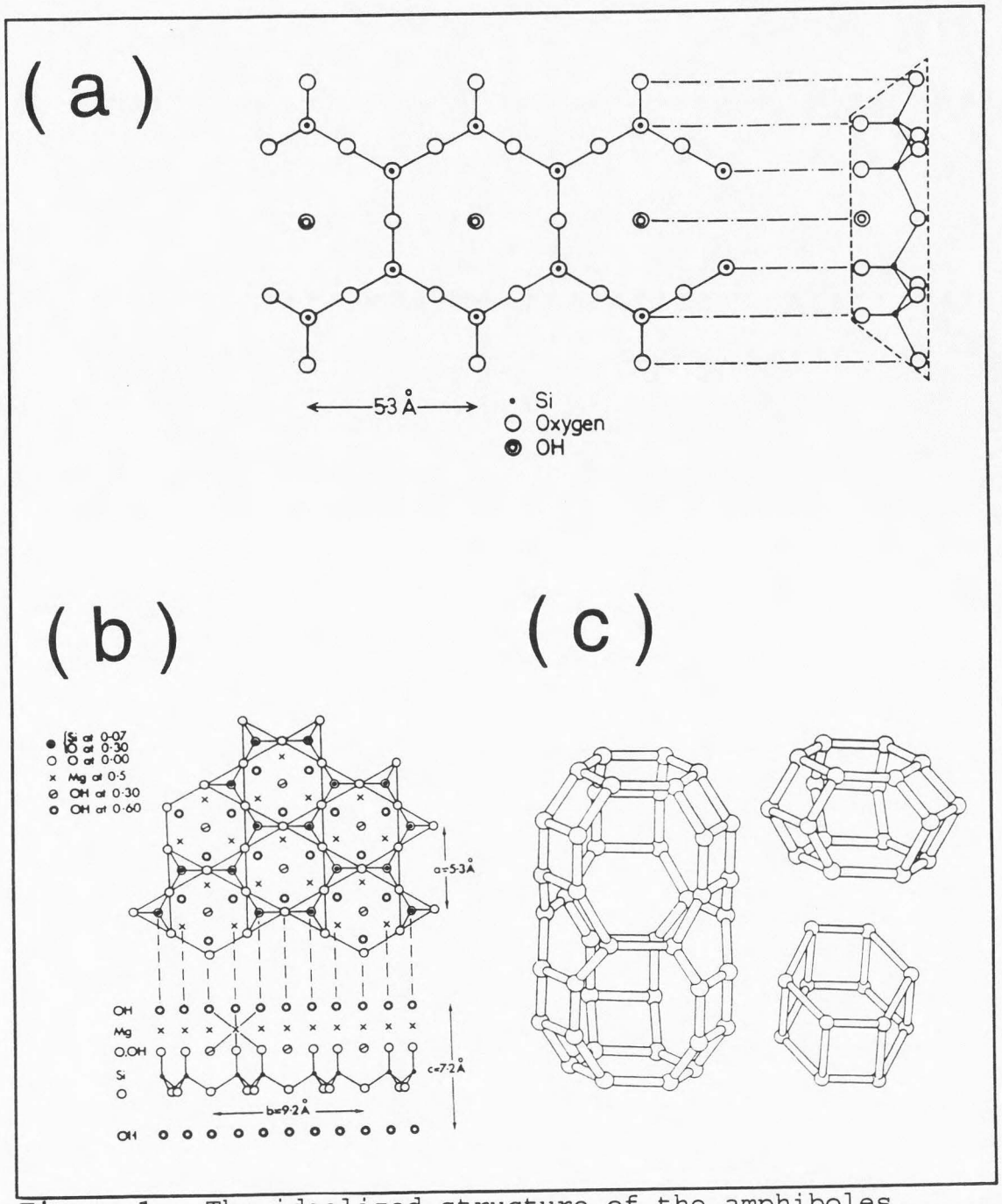

Figure 1. The idealized structure of the amphiboles, chrysotile and erionite. The silicate chain structure of the amphiboles (a), chrysotile (b), and the three dimensional structure of erionite (c).

one-dimensional sheets of silicon-oxygen tetrahedra.[22]

Sandwiched between the sheets are cations held in place by

van der Waals forces. Chrysotile (Figure 1b) is also composed of one-dimensional sheets of silicon-oxygen tetrahedra, but is made of overlapping sheets of the silicon-oxygen tetrahedra, and brucite, which is $Mg(OH)_2$. In chrysotile, the magnesium ions can be substituted with iron, which accounts for the iron content of the mineral. The sheets comprising chrysotile coil together to form scroll-like tubes with the magnesium-hydroxide surface facing outward. Small fibrils of chrysotile aggregate together into bundles which are known to break down and disseminate in aqueous suspension.

Erionite, like all zeolites, is an aluminosilicate composed of silicon-oxygen tetrahedra which associate into six-membered rings to form channels and cages, as shown in Figure 1c. Occasional substitution of Al atoms for Si gives rise to an overall negative charge on the molecule, which allows counter-cations to associate with the mineral. Cations can migrate into the 4.3 Å openings of the network of cages. This open cage-like structure gives erionite a surface area that is up to 50 times greater than the surface areas of asbestos fibers.

III. Physical Properties of Asbestos Involved in Carcinogenesis

Several of the characteristics and the empirical formulas of carcinogenic mineral fibers discussed in this review are compiled in Table 1. Many of the reactions that

will be discussed in this review have been reported to be strongly influenced by the surface area and surface silanol group coverage of the various mineral fibers. The surface coverage of silanol groups, as listed in Table 1, are by far the greatest in the asbestos minerals. Amosite has the greatest number of terminal OH groups, followed by crocidolite, chrysotile, and finally erionite.[23,24] When comparing the surface area, erionite has the greatest by far, 10-100 times larger than the asbestos fibers. Finally, the intrinsic iron content is greatest in the amphibole forms of asbestos, crocidolite and amosite. The iron in amosite is exclusively of Fe(II) while crocidolite contains Fe(II) and Fe(III).

Two properties of the asbestos fibers, size and durability, appear to affect their carcinogenicity. The first, and perhaps most familiar, is fiber size, or what has been termed "aspect ratio." For some years Stanton's hypothesis, that fiber size is the determining factor in fiber carcinogenicity, was widely accepted.[25] A good correlation has been reported between the induction of pleural mesothelioma in rats and the number of long, spindle-shaped fibers with 0.25 μm diameter and 8 μm length or longer residing in the lungs.[26] This subject has been previously reviewed and will not be discussed at length here.[27] However, aspect ratio does appear to be important because only respirable, durable fibers which are not small

Table 1. Physical Properties of Crocidolite, Amosite, Chrysotile, and Erionite

Mineral Fiber	Surface Area (m^2/g)[33]	Iron Content (% by weight)[9]	Surface Silanol Groups (groups/nm^2)[23]
Crocidolite $Na_2Fe^{(III)}_2(Fe^{(II)},Mg)_3Si_8O_{22}(OH)_2$	2-15	27.3%	4.7±0.6
Amosite $(Fe^{(II)},Mg)_7Si_8O_{22}(OH)_2$	1-6	28.5%	7.6±1.8
Chrysotile $Mg_3[Si_2O_5](OH_4)$	10-27	0.7%	1.8±0.1
Erionite $NaK_2MgCa_{1.5}(Al_8Si_{28}O_{72})*28H_2O$	354±9[a]	ND	0.0042-0.039[24]

[a] Johnson, N.R.; Hoover, M.D.; Thomassen, D.G.; Cheng, Y.S.; Dalley, A.; Brooks, A.L. *Am. J. Ind. Med.* **1992** *21*, 807-823.

ND Not detectable

enough to be cleared from the lung will remain for periods of time sufficient to cause disease. For example, Mossman has shown that riebeckite, which is chemically similar to the most carcinogenic form of asbestos, crocidolite, is not as toxic to cells in culture[28] and does not induce squamous metaplasia as crocidolite does.[29] The difference in the carcinogenicity of the two fibers may be attributed to the fibrous nature of crocidolite, which slows clearance from the lung, compared with the nonfibrous riebeckite, which may be cleared more easily. Goodglick and Kane[30] observed that when clearance of fibers was prohibited by repeated administration, short fibers produced the same cytotoxic response as long fibers. Notions about the aspect ratio of fibers cannot fully account for the chemistry of, nor the biological reactions to, mineral fibers. Recently the limitations of the utility of the Stanton hypothesis in accessing the carcinogenic potential of mineral fibers have been enumerated.[31,32]

A second property that appears to influence carcinogenesis is fiber durability; generally, the longer the fiber resides in the lung, the more likely it is to be carcinogenic. Amphibole fibers remain in the lung for the lifetime of the individual. Chrysotile has historically been used in 90% of the applications of asbestos,[33] but is much less carcinogenic than crocidolite or amosite. This may be due to the more rapid dissolution kinetics of the

serpentine minerals compared with the amphiboles. Although few studies have been performed to quantify the dissolution of mineral fibers, it is generally believed that chrysotile dissolves at a significantly greater rate than the amphiboles. Hume and Rimstidt[34] incubated chrysotile in aqueous solutions similar to human lung fluids and observed rapid dissolution of the fibers. In comparing chrysotile, crocidolite, and amosite, fibers were incubated in a severe environment of 4M HCl and refluxed to encourage dissolution. Sixty percent of the chrysotile was lost in 30 min while only 6% of crocidolite, or 8% of amosite dissolved in the same period of time. After 8 h of this treatment, 8.5% of the crocidolite fibers had been dissolved, while 30% of the amosite fibers dissolved.[35] This difference in dissolution between the amphibole and serpentine minerals may help to explain why the amphiboles are highly carcinogenic and chrysotile, is at best, weakly carcinogenic.[3] Studies on the dissolution of mineral fibers have been reviewed by Morgan and Holmes.[36]

IV. Reactions Catalyzed by Iron

Iron is by far the most abundant transition metal in the body. The average male has approximately 4 g of iron in his body while the average female body contains 3 g. The amount of iron is greater than the combined amounts of zinc, copper, molybdenum, cobalt, and all other trace metals in

the body.[37] Hundreds of proteins are known to contain iron atoms, including proteins, which transport oxygen, allow the synthesis of proteins and nucleic acids, produce energy through oxidative phosphorylation, and facilitate a multitude of other reactions for growth and reproduction. Although iron is essential for all life, if uncontrolled, it has the potential to catalyze the oxidation of DNA, lipid, and protein. Living organisms have evolved proteins to transport and store iron in an unreactive form until it is needed.[38] When iron is absorbed from the diet, it is bound to transferrin for transport in the blood. Transferrin receptors are synthesized and incorporated into the plasma membrane for binding and endocytosis of transferrin in cells which need iron. In a process that is not completely understood, iron is passed from transferrin to ferritin, where it is stored until needed. The reduction potential of these protein complexes with ferric iron is too negative to be reduced by typical intracellular reductants such as ascorbate, cysteine, or glutathione, which probably explains the stability of these protein-iron complexes *in vivo*.[39]

There are diseases in which iron may be observed bound to compounds other than protein. Hemochromatosis is a genetic disease which results in the abnormal accumulation of iron to high levels in the body. Under these circumstances, citrate-chelated iron has been observed in the blood.[40,41] Patients with hemochromatosis[42,43] or another

iron overload disease, porphyria cutanea tarda,[44,45] are more likely than persons without these conditions to develop liver cancer. The unusual appearance of citrate-chelated iron in the blood of patients with iron overload conditions may be involved in the symptoms observed in these individuals through the iron-catalyzed generation of oxygen radicals.[40,46,47] More recently it has been noted that high levels of iron in the body are associated with an increase in all types of cancers.[48-50]

A. Generation of Reactive Oxygen Species

Reactions of many biomolecules with molecular oxygen to generate reactive oxygen radicals do not occur because most organic molecules exist in the singlet spin state while O_2 exists in the triplet spin state. Reactions of a triplet with a singlet molecule are formally forbidden and will generally be slower than $10^{-5}M^{-1}s^{-1}$. However, transition metals like iron can bridge this kinetic restriction by reducing O_2 to form radical species that are capable of reacting with organic molecules.[39] Other transition metals with a free coordination site could catalyze the reduction of O_2 or H_2O_2. Although the exposure to other transition metals is generally low, they are capable of catalyzing deleterious reactions when introduced into biological systems.[51] Since iron exists at the highest concentration of any transition metal in most living organisms, it is thought

to be responsible for most of the abnormal oxygen radical production observed. Many mechanisms have been proposed as the potential reactions of iron to generate reactive oxygen species. The following is a series of reactions of iron which together lead to the generation of the hydroxyl radical.[52] These reactions are the modified, iron-catalyzed Haber-Weiss reactions.

$$\mathbf{Reductant^{(n)} + Fe(III) \rightarrow Reductant^{(n+1)} + Fe(II)} \quad (1)$$

$$\mathbf{Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\cdot -}} \quad (2)$$

$$\mathbf{HO_2^{\cdot} + O_2^{\cdot -} + H^+ \rightarrow O_2 + H_2O_2} \quad (3)$$

$$\mathbf{Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {\cdot}OH} \text{(Fenton Reaction)} \quad (4)$$

Hydrogen peroxide, which reacts very sluggishly with biomolecules, and $O_2^{\cdot -}$, which reacts with second-order rate constants ranging from 2.3×10^4 to 2×10^9 $M^{-1}s^{-1}$, are not considered to be damaging species in biological systems.[53] The ·OH reacts with most biomolecules with second-order rate constants ranging from 2×10^8 to 3.6×10^{10} $M^{-1}s^{-1}$ which are considered diffusion controlled reaction rates.[53] Thus, it is the production of ·OH or a similarly reactive species that is generally considered to be dangerous to the cell.[54] Therefore, the important intracellular reactions are those that generate Fe(II) and H_2O_2. Ferrous iron may already be present or may be generated by the reduction of Fe(III) by ascorbate, cysteine, glutathione, or other cellular reductants. Activated macrophages can produce $O_2^{\cdot -}$, which is also capable of reducing iron, under unusual

conditions.[39,55] Hydrogen peroxide may come from an exogenous source, such as activated macrophages, or be generated by the spontaneous or enzyme-catalyzed dismutation of $O_2^{\cdot-}$. Iron is a catalyst in these reactions and will continue to redox cycle, producing $^{\cdot}OH$ as long as there is sufficient reductant and either O_2 or H_2O_2. Redox cycling of iron may lead to significant damage to biomolecules in a manner similar to X-rays or γ-rays, where the $^{\cdot}OH$ is thought to be responsible for the induction of cancer.[56]

Pryor has suggested that the hydroxyl radical is the predominant damaging species *in vivo* because the elements which generate the hydroxyl radical, such as iron, are often capable of binding to the DNA itself and allow $^{\cdot}OH$ production to occur in the immediate vicinity of the DNA.[57] For radicals to damage DNA they must not only be thermodynamically favored to do so, but must also have the kinetic energy to reach the site of damage and to react.[57] Other radicals, like carbon-centered radicals, are known to react readily with nucleic acids *in vitro*, but excreted methyl radical adducts are observed with a much lower frequency than hydroxyl radical adducts, although their predicted reactivities are similar. Pryor has suggested that the most electrophilic radical that DNA is generally exposed to is the $^{\cdot}OH$. The combination of these three characteristics, high electrophilicity, high thermokinetic reactivity, and a mechanism for production near DNA, makes

the hydroxyl radical the only radical which generally damages DNA bases.[57]

The reactivity of iron is highly dependent upon its electronic environment. For example, iron bound to low-molecular-weight chelators like citrate, adenosine diphosphate (ADP), or ethylenediaminetetraacetic acid (EDTA) is redox active.[39,46,58] Graf *et al.*[55] have shown that coordination of iron by these chelators allows water, or other small molecules like O_2, to occupy an available coordination site. This allows the iron chelates to reduce O_2 and generate highly reactive species.[55] In contrast, when all of the iron coordination sites are tightly bound by the chelator, excluding other molecules, the complex will be redox inactive, as occurs with N-[5-[3-[5-(X)hydroxy-acetamide)pentyl]carbamoyl] propionohydroxamic acid monomethanesulfonate (desferrioxamine B)[55] and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p-sulphonic acid (ferrozine), which stabilize the Fe(III) and Fe(II) forms, respectively. Inhibition of chemical reactions or biological effects by desferrioxamine B has been extensively used to determine whether reactions are catalyzed by iron. Although desferrioxamine B is generally considered to bind Fe(III) preferentially [log K of 31] over Fe(II) [log K of ~7], it is known to coordinate both oxidation states.[53,59,60]

The location of iron within the cell also influences potential reaction since the ·OH generated by the iron-

catalyzed reactions is diffusion-limited in its reaction kinetics. Thus, iron must be within 10 Å of the target molecule in the cell for damage to occur. This restriction will become very critical when asbestos-catalyzed reactions are discussed.

B. Oxidation of Biological Molecules

The reactions of iron with biological molecules have been extensively studied. There are many proposed reactive species resulting from interaction with iron. In addition to generation of ·OH, the ferryl iron species, Fe(IV)=O and Fe(II)-Fe(III)-O_2 complex have been proposed to be involved in reactions catalyzed by iron. Of all of the reactive species generated by iron, the ·OH radical is the only species that has been extensively studied, probably because methods are more readily available for its detection and participation in reactions.

Since the short-lived ·OH cannot be detected directly, electron paramagnetic resonance spectroscopy (EPR) is used to detect the formation of a more stable radical species resulting from reaction of spin-trapping chemicals with ·OH. To determine whether ·OH is responsible for the oxidation of biological molecules, antioxidant enzymes, such as superoxide dismutase (SOD), which catalyzes the dismutation of $2O_2^{\cdot-}$ to H_2O_2 and H_2O, or catalase, which causes the decomposition of H_2O_2 to H_2O and O_2, are often used. By

reexamining Reactions 1-4 discussed previously, one can see that introduction of SOD into a reaction system which is generating ·OH should increase the rate of formation by increasing the rate of generation of H_2O_2, Reaction 3. In contrast, catalase should inhibit the formation ·OH by removing H_2O_2 and inhibiting Reaction 4. Further evidence for the participation of ·OH in reactions is to determine whether radical scavengers, such as 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO), salicylate, mannitol, ethanol, dimethyl sulfoxide, or dimethylthiourea, inhibit the formation of products being monitored. All of these methods are fairly easily used for *in vitro* reactions, but become much more difficult to implement and interpret *in vivo* or in cultured cells.

1. DNA Damage

Iron appears to induce the same types of damage to DNA that γ irradiation does through the generation of the ·OH.[61] Iron-catalyzed reactions with DNA

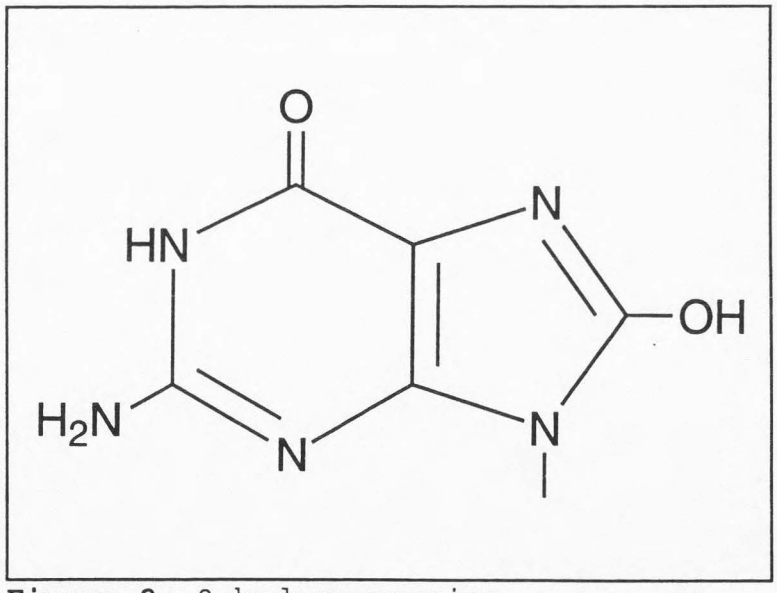

Figure 2. 8-hydroxyguanine

result in damage to all four bases and the deoxyribose, which is manifest as strand breaks to DNA. To quantify and identify the many products produced, Dizdaraglu and co-workers have used gas chromatography/mass spectroscopy with selected-ion monitoring. Seventeen different modified base products have been reported using this technique.[56] The predominant product observed is 8-hydroxy-2'-deoxyguanosine (8-OHdG),[62] the structure of which is shown in Figure 2. They detected an enhancement in the amount of adducts from EDTA:Fe(III). The production of hydroxylated bases was inhibited by the addition of antioxidant enzymes, radical scavengers, or the inactivating chelator, desferrioxamine B,[63] suggesting that iron-catalyzed Haber-Weiss chemistry may be responsible for the production of the adducts observed. They later showed that the chelator nitrilotriacetate (NTA) produced by far the greatest amount of these base products in the presence of H_2O_2.[47] This observation is of particular interest because NTA-chelated iron is a known carcinogen causing acute nephrotoxicity and renal carcinoma[64-66] and lipid peroxidation.[67,68] Mammalian chromatin was also susceptible to oxidative damage by iron. This effect was enhanced by addition of ascorbate and the investigator concluded that the damage was due to the hydroxyl radical formed by redox active iron.[62]

Floyd and co-workers have developed a more rapid and sensitive means of detecting this oxidized base product

using reversed phase HPLC with electrochemical detection.[69] Because of the preponderance of this base product and the ease and sensitivity of the HPLC assay, most laboratories investigating the participation of iron-catalyzed oxygen radicals in DNA damage are currently using this technique. Many of the observations which have been made on the redox chemistry of iron chelates, both *in vitro* and *in vivo*, have been recently reviewed.[51]

The relationship between the formation of modified DNA bases and the induction of cancer is not clear. Although 8-OHdG may not be directly responsible for carcinogenicity, its presence at elevated levels is a very good indication that abnormal oxidative reactions are occurring. Floyd has outlined what he considered to be a direct correlation between the presence of 8-OHdG and conditions leading to carcinogenesis.[70] He enumerated the following four lines of evidence supporting his proposal. First, ionizing radiation is known to cause cancer and induces 8-OHdG. Second, the strong oxidant $KBrO_3$ causes kidney tumors which contain elevated levels of 8-OHdG. NaClO or $NaClO_2$, which are equally strong oxidants, do not cause tumors or an increase in 8-OHdG in treated tissue. Third, treatment of rats with the Fe:NTA complex is carcinogenic in kidney and causes an increase in 8-OHdG in kidney DNA. The complex Na:NTA is not carcinogenic and does not cause elevated levels of 8-OHdG. Fourth, a liver carcinogen, 2-nitropropane, is associated

with increased levels of 8-OHdG, whereas the isomer 1-nitropropane, which is not carcinogenic, does not cause any increase in 8-OHdG formation upon administration. Floyd noted that these conditions reflected oxidative stress, and under carcinogenic conditions not involved in oxidative stress, the correlation was not observed.[70] Iron is known to generate many products which are associated with oxidative stress, but 8-OHdG may be a valuable indicator of the carcinogenic potential of various iron-containing compounds.

Iron-catalyzed oxygen radical production is also known to introduce strand breaks into DNA.[61,71] DNA strand-break assays can be highly sensitive to the reactions of iron when closed-circular, superhelical DNA is used as the target. DNA strand breaks have also been reported to occur in the presence of various chelates of iron in isolated cellular DNA of prokaryotic[52,72] and eukaryotic chromosomal DNA.[73] The specificity and location at which the damage to the DNA occurs appear to depend on the chelator to which iron is bound.[72,73]

The relationship between the types of DNA damage observed after exposure to iron and mutation is largely unknown. The presence of 8-OHdG in DNA has been shown to lead to misincorporation of bases during replication of DNA *in vitro*.[74] Loeb *et al.* observed mutations in ϕX174 DNA after exposure to iron and transfection into bacterial spheroplasts.[75] The investigators concluded that the types

of mutations observed were typical of those observed after reactions with oxygen radicals. The pattern of mutations was nonrandom, suggesting that these mutations occurred at specified locations.[76] A thorough review on metals in carcinogenesis has recently been published.[77]

2. Lipid Peroxidation

The role of iron in lipid peroxidation has been extensively studied and the identity of the oxygen species responsible remains controversial. A reactive oxygen species other than ·OH appears to be responsible for lipid peroxidation because catalase, superoxide dismutase, or mannitol did not inhibit iron-dependent lipid peroxidation. Several investigators have suggested that both Fe(II) and Fe(III) and O_2 are required for lipid peroxidation to occur. The maximal rate of lipid peroxidation was observed when Fe(II) and Fe(III) were available at a 1:1 ratio.[78-81] It has been proposed that a complex between Fe(II), Fe(III) and O_2 may be involved,[81] but no definitive evidence for this complex exists at this time.

Some of the products of lipid peroxidation, 4-hydroxynonenal, 4-hydroxyhexenal, and malonaldehyde, shown in Figure 3 have been extensively studied and are generally considered to be dangerous because of their long lifetimes and ability to traverse the cell. Their reactivity with biomolecules, such as proteins and nucleic acid bases, may

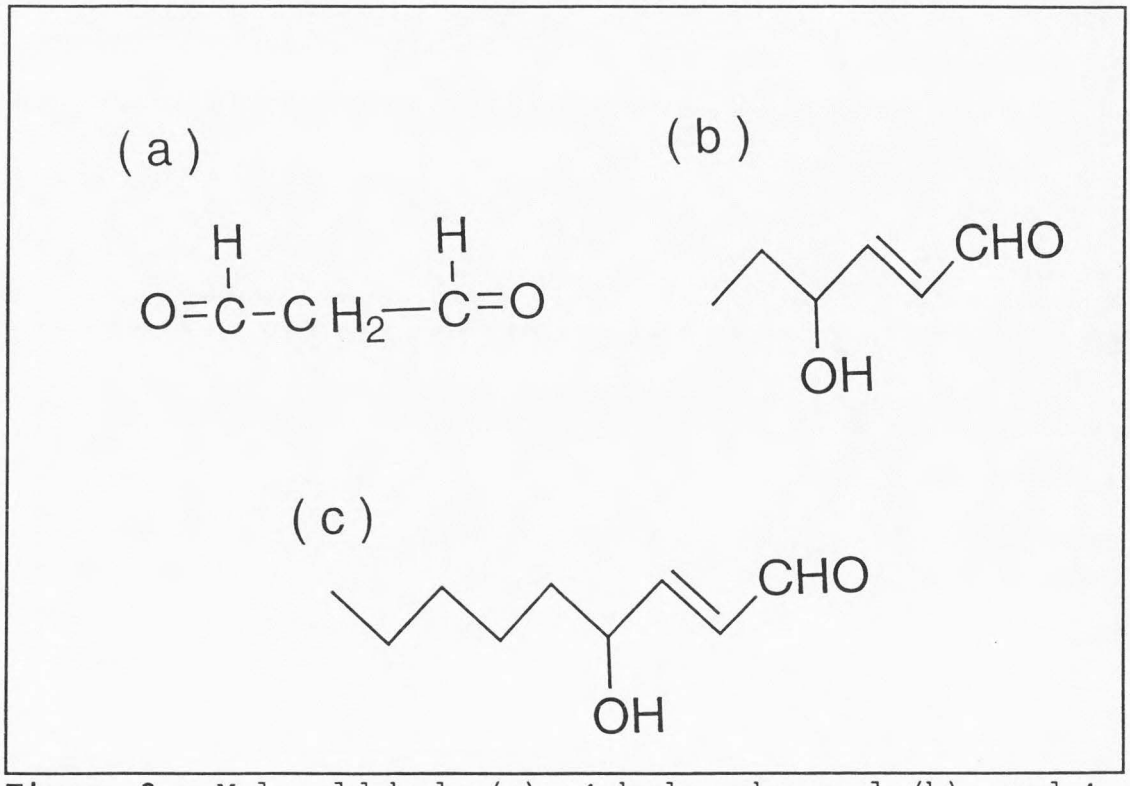

Figure 3. Malonaldehyde (a), 4-hydroxyhexenal (b), and 4-hydroxynonenal (c).

be involved in the initiation of cancer in the same way that damage to these biomolecules by radicals may be involved. This is the subject of a recent, comprehensive review.[82]

3. Protein Oxidation

Iron has also been implicated in the oxidation of proteins. Oxidation of proteins is thought to be significant in ageing,[83] but its role in the development of cancer is not clear. Oxidation of proteins, like glutathione or glutathione peroxidase, may lead to a compromised antioxidant defense system, and the oxidation of DNA repair enzymes at a critical time may allow mutations to occur.

Both Fe(II) and Fe(III), as well as iron-containing proteins and hemoglobin degradation products, have been reported to cause oxidation of individual amino acids as a result of iron-catalyzed reactions.[84] Although the reactive oxygen species responsible for this damage have not been confirmed, Stadtman has proposed a complex model for the involvement of both Fe(II) and Fe(III) and O_2 in the oxidation of proteins.[83,85]

C. Iron-induced Carcinogenesis

Iron may cause cancer as a result of oxygen radical-induced damage to DNA. There is increasing evidence that iron from sources other than asbestos can increase the risk of cancer in humans.[86-88] In rodents, the evidence is even more convincing. Iron-NTA has been shown to induce renal adenocarcinoma[89] and iron-dextran to induce sarcomas at the site of injection.[90] An even more significant observation was that intraperitoneal injections of iron-saccharate in rats caused mesothelioma,[89] the same type of rare tumor induced by asbestos. In all of these experiments with rodents, it was convincingly demonstrated that iron was required for the carcinogenic response.

V. Reactions Catalyzed by Asbestos

The surface character of mineral fibers determines their chemical reactivity, their ability to bind and release

elements, and their surface charge. These surfaces are not inert, but are dynamic and highly interactive with their environment. Fiber surfaces can be modified simply by milling, suspension in aqueous solution, or changes in temperature or pressure. The surface composition can be modified as a result of binding or releasing of atoms or molecules. The type of binding, adsorption in a monolayer or nucleation and formation of a 3-dimensional structure, is extremely important in determining chemical reactivity and binding and desorption kinetics. The surfaces can also have lateral heterogeneity, which plays an important role in mineral surface chemistry. Surface heterogeneity can arise during adsorption or desorption of elements.

Mineral surfaces are complex not only in terms of atomic structure and composition, but also in terms of microtopography.[91] It is clear that there are "active sites" on the surfaces which will facilitate adsorption or desorption. "Active sites" often occur where surfaces are rough or the microtopography is uneven. The atoms that make up the top of the rough edges or terraces have unsaturated coordination sites and therefore are capable of undergoing reactions.

Studies to understand the chemical reactivity, the ability to bind and release elements, and the surface microtopography of mineral fibers that pose health problems have increased in the past 10 years. In this section, we

will summarize what has been learned about these unique mineral fibers.

A. Surface Chemistry

In order for any chemical reaction to be catalyzed by solid fibers, *in vitro* or *in vivo*, interactions between the surface of the fiber and the environment must occur. Fubini and associates stressed that the reactivity of fibers *in vivo* will be controlled by the surface chemistry of the individual fibers.[92,93] Some of the surface chemical functionalities which are present on the surface of fibers are H-bonding sites, dangling bonds, poorly coordinated metal ions, charges which occur due to lattice vacancies and defects, unoccupied cation coordination sites, and Lewis acids or bases.[94]

1. Reactive Sites

Hochella has pointed out that, unlike the figures which are commonly drawn of mineral fibers (see Figure 1), the surfaces of most mineral fibers are not atomically flat, but contain atoms above the plane of the mineral and holes which were previously occupied.[91] The microtopography of the surface of even a very flat mineral might appear uneven on an atomic scale. This type of occurrence is magnified when minerals are weathered or milled, and mineral fibers develop additional areas of nonideal microtopography. Fractures occur along cleavage planes to create the edges of minerals

which are often considered to be the areas of greatest deviation from the idealized structure. These portions of the mineral are where dangling bonds, silanol groups, and unoccupied cation coordination sites may exist. Many of the reactions catalyzed by asbestos and erionite fibers may be strongly influenced by the surface functional groups made accessible both by the native structure of the mineral and by mechanical processes like milling or weathering, generating nonideal surfaces which may be the areas of the greatest reactivity.[91]

Responses of silicate surfaces to grinding have been compiled by Fubini *et al.*[95] Homolytic and heterolytic cleavage of bonds leads to stuctures like those in Figure 4: distorted siloxane bridges, peroxide bridges, silica radicals, Si^+ or $Si\text{-}O^-$ surface charges, $Si\text{-}O_2^{\cdot}$ peroxyradical, and $Si^+O_2^{\cdot-}$ superoxide radical, which have been observed on silicate surfaces after mechanical grinding.[95] These surface functional

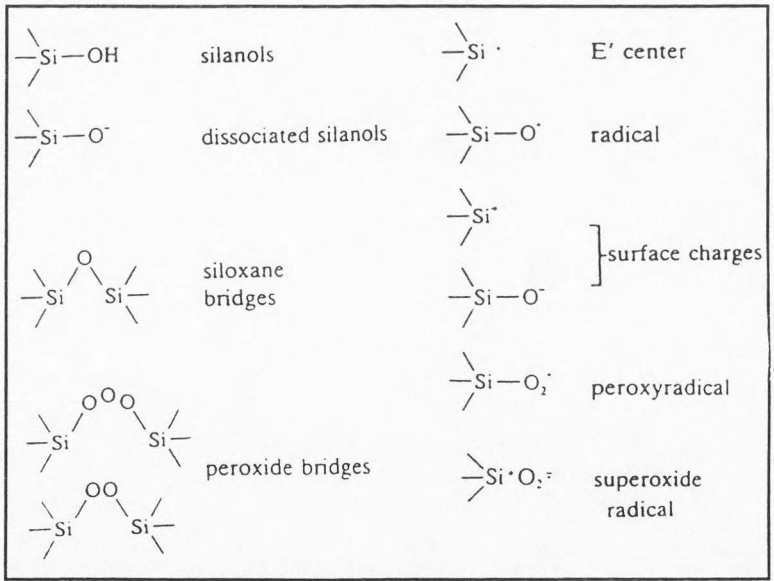

Figure 4. Surface functional groups commonly found on silicate minerals.

groups may play a role in freshly ground silicates, but are rapidly lost by heating or by dissolution in aqueous media, which convert silanols (hydrophilic surfaces) to siloxanes (hydrophobic surfaces). These surface reactive groups would be dissipated shortly after inhalation of fibers into the moist environment of the lung. While these groups may be involved in acute biological effects, the long-term contribution of these surface reactive groups to carcinogenicity is questionable.[92]

2. Redox Active Iron

The long-term reactivity of the surface of asbestos fibers may be largely governed by the amount of iron which is coordinated to the surface and the redox activity of that iron. Shen *et al.*[96] have developed a novel way to measure the reactive iron on the surface of iron-containing mineral fibers. This electrochemical method utilizes soluble mediators to carry electrons between the solid electrodes and the solid sample, which is contained within a thin-cell of 25 μm thickness. Crocidolite and amosite were examined using this mediated, thin-layer cell, coulometric method. The total amount of redox active iron on the surface of crocidolite was 4.3 $\pm$ 0.7 nmol Fe/mg, whereas amosite contained 3.3 $\pm$ 0.7 nmol Fe/mg. The surface coverage of iron on the two minerals was of course dependent on the surface area of the mineral fibers themselves. Crocidolite

held 4.3 x 10^{-11} mol Fe/cm^2 and amosite had 9.5 x 10^{-11} mol Fe/cm^2. Seventy-six percent of the redox active surface iron on crocidolite was in the Fe(II) form while only 25% of that on amosite was in the reduced form.[96]

These results are in agreement with previous findings on the oxidation state of mobilized iron from crocidolite using an iron chelator, ferrozine, and ascorbate as a reductant.[97] This study demonstrated that 66% of the iron mobilized from crocidolite was in the Fe(II) state, while only 10% of iron mobilized from amosite was reduced. More iron was mobilized than the amount of surface reactive iron determined by the electrochemical method, but the ratio between the oxidation states was similar.[96,97] In the mobilization experiments, it is likely that the greater quantity of iron observed may have come from the outer layers rather than exclusively from the surface *per se*. An important outcome of these electrochemical experiments is that they showed that the iron can be repeatedly oxidized and reduced. This may be very important in the reducing environment of the cell where O_2 is also present. Many of the investigators studying iron associated with mineral fibers are most concerned about the Fe(II) content. This may be inconsequential compared with the total iron present if the iron can redox cycle.

3. Hydroxyl Radical Generation

Weitzman and Graceffa[98] were the first to actually study the surface reactivity of fibers. They observed that chrysotile, crocidolite, or amosite, suspended in aqueous solution with H_2O_2, generated ·OH, observed by EPR after spin trapping with DMPO. The addition of desferrioxamine B to the reaction mixture inhibited the ·OH-DMPO adduct signal, indicating that the reactivity was due to iron on the various fiber surfaces.[98]

The spin adducts observed in these experiments were in an environment free of iron chelators, suggesting that the observations made were the result of surface iron. The work to be discussed now reported studying surface reactivity. However, the experiments were performed in the presence of buffers, such as phosphate or Tris, which are capable of mobilizing iron from asbestos into solution.[52]

Pezerat has proposed that there are two types of reactive oxygen species generated by iron which are responsible for the fact that some fibers induce predominantly bronchial carcinoma while others induce predominantly mesothelioma.[99] He suggests that A^*, a strongly oxidizing species capable of hydrogen atom abstraction, e.g. ·OH or Fe(V)=O, is capable of direct attack on the genome and is not affected by antioxidant enzymes. The second P^* group of species is comprised of all other types of oxidizing species and is, he proposes,

responsible for activities such as lipid peroxidation, the products of which he proposes lead to mesothelioma. Erionite, for example, was not capable of generating A^* species and erionite is very efficient at inducing mesothelioma, but only rarely causes lung cancer. The more reactive A^* species are proposed to be responsible for causing bronchogenic carcinoma, and to be detected, required an environment rich in phosphate to accelerate the leaching of inorganic solid phases.[99] DMPO was employed to assay various mineral fibers for their ability to form ·OH radical adducts for EPR analysis, and a linear correlation was observed between the oxidizing surface activity and the Fe(II) content of the fiber.[100] Although the work is referred to as surface reactivity, reactions of the A^* sites may occur due to mobilized components from the fibers, since phosphate buffer was required for reactions to be observed.

Pezerat and co-workers have also suggested that the preliminary step in the generation of reduced oxygen species occurs when H_2O binds to a Fe(II) on the surface of a fiber.[101] In this model, attack of an O_2 results in oxidation of the iron with binding of hydroxide while H^+ and $O_2^{\cdot-}$ are released.[101] No evidence for this mechanism was given, and a scheme such as this would require subsequent interaction of more Fe(II) to generate the further reduced oxygen species they have observed. The details of this mechanism may be garnished from a previous study which noted that

coordination by H_2O allowed reduction of O_2 to $O_2^{\cdot-}$ only after the H_2O was displaced by O_2 in the proper coordination site.[55]

A comparison of the ability of a fiber to generate $^{\cdot}OH$ and the formation of 8-OHdG also yielded a very strong correlation.[102] Grinding was used to increase the oxidizing potential of minerals. After grinding, amosite or crocidolite had a 145- or 125-fold increase in ability to generate DMPO adducts and a 53- or 22-fold increase in ability to catalyze the formation of 8-OHdG, respectively. Erionite was only slightly capable of generating DMPO adducts before or after grinding, and there was a meager 5-fold increase in the ability of ground erionite to generate 8-OHdG. This was interpreted as evidence for the P^* species before grinding and A^* species in crocidolite and amosite after grinding.[102] Grinding appears to expose more new surfaces with Fe(II), since reducing agents were not used in these experiments. An alternative explanation for the apparent discrepancy between the biochemical inactivity of erionite *in vitro* and its potent carcinogenic activity *in vivo* will be discussed at some length in the section on iron binding to erionite.

In work by Fubini *et al.*, fibers were treated with ferrozine, a chelator with a high affinity for Fe(II), or with desferrioxamine B, which is a chelator for Fe(III) predominantly.[92] In either case, treatment with the chelator resulted in a loss of free radical release, which was

detected using EPR with the spin trap DMPO. The investigators proposed that both Fe(II) and Fe(III) species are required for the generation of free radicals through the reduction of O_2. A proposal of the required conformation of Fe(II) and Fe(III) on the surface of fibers as described by this research is shown in Figure 5. This figure depicts how both oxidation states of iron are present both in and on the surfaces of fibers and are able to react with small molecules like O_2 and H_2O_2 to generate activated oxygen species. Desferrioxamine B has been shown to bind to crocidolite.[103] The investigators suggested that the loss of activity observed after chelator treatment may have been a result of the chelators obstructing the normal interactions with the surface of the mineral fiber, preventing the Fenton reaction.[93] Another reason for loss of reactivity may be explained on the basis of removal of iron from the catalytic sites on the fibers. This will be discussed in more depth in the section on mobilization of iron.

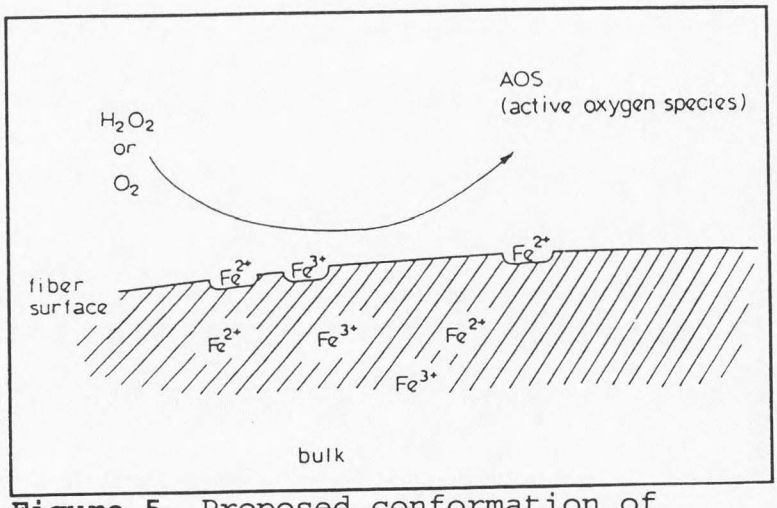

Figure 5. Proposed conformation of Fe(II) and Fe(III) on the surface and in interior of mineral fibers. Iron on the surface allows generation of active oxygen species.

Ghio *et al.*[23] examined the role of surface iron in the ability of crocidolite, suspended in phosphate buffer with H_2O_2 and ascorbate, to catalyze the formation of ·OH. The formation of ·OH was assessed by measuring the formation of thiobarbituric acid reactive products from DNA and lipid and by measuring the hydroxylation of salicylate. These investigators defined surface iron to be the iron which was mobilized in 30 min into a solution containing citrate, bicarbonate, and dithionite at 70°C. Crocidolite, pretreated with increasing concentrations of the iron-chelator desferrioxamine B, became less capable of generating ·OH. They concluded that the amount of ·OH produced was proportional to the amount of Fe(III) on the surface of the fibers.[23] These results differ from those of Pezerat, who observed that Fe(II) rather than Fe(III) appeared to be related to the ability of fibers to generate ·OH.[100] The electrochemistry studies[96] and the reports by Fubini *et al.*[92] which were discussed previously both observed Fe(II) as well as Fe(III) on the surface of crocidolite fibers. There may be two reasons for the conflict. First, an inherent assumption in these experiments was that desferrioxamine B only binds Fe(III). However, desferrioxamine B is known to bind both Fe(II) and Fe(III),[53,59,60] so both ions may have been bound. Second, the reducing agent ascorbate was used, which makes it difficult to differentiate between Fe(II) and Fe(III) reactivity.

Despite the difficulties in interpretation, a relationship between the total surface iron and the ability to generate the ·OH was observed here.

In order for chemical reactions to occur between a solid, like a fiber, and an aqueous environment, such as exists in the cell, surface interactions must take place. Many of the results discussed here have suggested the transfer of an electron from iron on the surface of asbestos to O_2 to generate reactive oxygen species. Generation of ·OH on the surface of asbestos is only important in reactions with biomolecules when the asbestos fiber is within approximately 10 Å of the target biomolecule because of the diffusion-controlled reaction kinetics of ·OH. Therefore, DNA damage from surface generation of ·OH is only possible when the fiber is in the nucleus of the cell. However, fibers are seldom observed in the nucleus. Unscheduled DNA synthesis[104] and DNA single-strand breaks (SSBs)[105] have been observed in asbestos-treated, cultured cells when no fibers were observed in the nucleus. Therefore, the surface of fibers may be more important in regulating the binding and release of other molecules, like iron, than in generating reactive oxygen species directly.

IV. Iron mobilization

Phagocytosis of asbestos fibers may constitute an uncontrolled entry of iron into the cell, since the fibers

have bypassed control by the protein transferrin. If iron can be mobilized from the fibers by low-molecular-weight chelators, such as citrate, the redox activity might be altered, and the chelate complex could diffuse throughout the cell and have the potential of catalyzing the formation of ·OH to damage DNA.

Mobilization of iron into solution has often been detected using the chelator ferrozine, which forms a colored complex of high extinction coefficient with Fe(II).[106] Mobilization of Fe(II) by ferrozine can be determined directly by quantifying the amount of ferrozine-Fe(II) complex formed. With other chelators which do not form complexes with high extinction coefficients, asbestos is incubated in solutions containing the chelator for varying periods of time, the asbestos is removed, and the iron mobilized by the chelator is quantified using a total iron assay with ferrozine.[52] Other chelators that have been used by investigators to detect and quantify iron mobilization are desferrioxamine B, which forms a colored complex with Fe(III), and EDTA, NTA, and citrate, which bind both oxidation states of iron. Several investigators have used chelators such as these to determine the rates of iron mobilization from various forms of asbestos and other minerals and to determine the factors which affect mobilization.

1. Factors Influencing Mobilization

Aust and co-workers have studied mobilization of iron from crocidolite, amosite, chrysotile, and erionite *in vitro*. These studies have revealed that mobilization requires the presence of a chelator at physiological pH.[52] This suggests that leaching of iron from asbestos, which has been reported *in vivo*,[107-111] must be the result of chelation. They have also found that the rate of mobilization depends upon the chelator being used.[52] Table 2 lists some of the chelators which have been used for mobilization studies and some of the experiments examining the catalytic activities of crocidolite fibers. In reviewing this table, it is clear that the stability constants of the chelators do not correlate well with the rate of mobilization of iron from the fibers. Other factors influencing mobilization may be the geometry and size of the chelator and the complementarity of its coordination of iron with that of the fiber.

When using these chelators to study the effects of mobilization on reactivity of iron, one must also take into account the effect of the chelator on the reactivity of iron. This is best illustrated by examining the reactivity of iron chelates in solution without the added complication of mobilization. The ability of iron chelates to produce reactive oxygen species, detected as ·OH formation or 8-OHdG, does not correlate with the stability constants of the

Table 2. Effect of Iron Chelators on Reactivity of Iron in Solution and on Crocidolite

Chelator	Stability Constant[a] (log K_a) Fe(II)	Fe(III)	Rate of Iron Mobiliztion from Crocidolite (nmol/mg/h)	Crocidolite-dependent DNA SSBs[c] (percent)[46]	Fe-dependant ·OH Formation[b] (nmols HCHO/ 30 min)[55]	Fe-dependent 8-OHdG formation[b] (per 10^5 dG)[47]
Desferrioxamine B	--	30.7	12.4±1.7[d]	ND	ND	--
EDTA	14.3	25.0	30.1[52]	29 ± 10	16.1 ± 0.3	1.43 ± 0.31
NTA	8.3	15.9	29.2[97]	61 ± 3	23.8 ± 0.3	13.40 ± 0.03
Citrate	4.4[e]	--	4.2±0.5[52]	2 ± 3	--	0.71 ± 0.07

[a] Martell, A.E.; Smith, R.M. *Critical Stability Constants*, Vol. 1; Plenum Press: New York, 1974.
[b] Incubated with solutions of iron in the presence of the indicated chelator.
[c] Expressed as the percent of closed-circular DNA with SSBs after incubation with crocidolite.
[d] Werner, A.J.; Hochella, M.; Rimstidt, J.D.; Aust, A.E.; Hardy, J.A. *Am. Mineralogist* **1995** in press.
[e] Dawson, R.M.C.; Elliott, C.C.; Elliot, W.H.; Jones, K.M.; *Data for Biochemical Research, 3rd edition*, Oxford University Press: New York, 1986, p. 400-413.
ND Not Detectable

complexes (Table 2). Thus, in examining the ability of crocidolite to induce DNA SSBs in the presence of various chelators, the results reflect differences between the chelators in their abilities to mobilize iron and to catalyze the formation of oxygen radical species. With desferrioxamine B, the coordination complex formed with Fe(III) makes the reduction of iron very difficult,[55] and the desferrioxamine B complex was unable to catalyze the formation of DNA SSBs, although iron was mobilized from the fibers. In the case of EDTA and NTA, iron was mobilized at approximately the same rate from crocidolite, yet NTA had the greater ability to catalyze the formation of DNA SSBs (Table 2). This correlated with the greater ability of NTA chelates to facilitate the formation of reactive oxygen species, followed by EDTA and citrate. These experiments stress that the reactivity of iron mobilized *in vivo* from asbestos fibers will depend upon the chelator involved. They also suggest that mobilized iron, rather than surface complexed iron, was responsible for the DNA SSBs observed. Experiments have been done to address this issue in more detail and will be discussed in the following sections.

When comparing mobilization of iron from different mineral fibers, factors which may be important are the crystalline structure, the surface area, and the iron content of the fibers. Crocidolite and amosite have very similar iron contents. When mobilization rates with either

ferrozine or citrate were compared, iron was mobilized more rapidly from crocidolite than amosite on a weight basis.[52] However, when corrections were made for the difference in surface areas, the rates were approximately the same for these two amphiboles.[52] Very little iron was mobilized from long or short fiber chrysotile[52] and no iron could be mobilized from erionite.[112] For the iron-containing fibers, the amount of iron mobilized was consistent with the observed carcinogenicities of the various fibers and was representative of the iron content.

The most carcinogenic forms of asbestos can reside in the lungs for decades. Therefore, it may be possible that iron would be removed from these fibers over long periods of time. To determine the effect of long-term removal of iron from crocidolite or amosite, fibers were incubated in aqueous solutions of desferrioxamine B for varying times up to 15 days.[113] The rate of iron mobilization decreased with time, and, after 15 days, totals of 215 or 70 nmol Fe/mg fiber were removed from crocidolite or amosite, respectively. As more iron was removed from the crocidolite or amosite fibers by desferrioxamine B during this time, less iron was available for subsequent mobilization by citrate or EDTA, and fewer DNA SSBs were observed.[113] This is similar to the observation of Ghio *et al.*,[23] discussed in an earlier section, that short-term desferrioxamine B treatment of crocidolite resulted in a decrease in oxygen

radical formation. Longer term incubations (90 days) showed that iron could still be mobilized from both forms of asbestos by desferrioxamine B, even after that prolonged period of time.[113] If iron is responsible for the cytotoxicity of fibers, these results would predict that desferrioxamine B treatment would reduce, but not eliminate the toxicity of these fibers to cultured cells. This is consistent with what has been reported for several different cell types.[30,114-120]

The effect of iron mobilization on the structure of asbestos has been studied by Mollo *et al.*[93] High resolution transmission electron micrographs of crocidolite and amosite fibers that have been incubated either in water or in desferrioxamine B to remove iron have been taken. The outer layer of the desferrioxamine B-treated fibers had become amorphous, probably due to removal of cations. However, the overall silicate structure appeared to be intact. This suggests that as iron is mobilized, cation-binding sites are vacated, which may allow subsequent occupation by cations from solution.

Other factors that have been shown to influence mobilization of iron from asbestos *in vitro* are temperature, pH, and time in aqueous suspension. Mossop[121] has shown that the initial rate of iron mobilization from crocidolite by EDTA was diminished 30% after 30 min incubation in aqueous suspension at room temperature or by 85% after 30 min of

autoclaving at 125°C in aqueous suspension. Autoclaving the dry fibers had no significant effect on the rate of iron mobilization from the fibers. This could have important implications, especially for *in vitro* biochemical studies, where suspensions of fibers are allowed to sit for some time before use, or studies in cultured cells, where autoclaved fibers are often used. The pH of incubation also appears to be important in iron mobilization from fibers.[52,113] The rate of iron mobilization from crocidolite or amosite by desferrioxamine B was consistently greater at pH 5 than at pH 7.5.[113] The investigators concluded that this may be relevant to physiological conditions, since fibers are often observed in phagosomes of cells where the pH can be 5.[113]

The oxidation state of the iron on the surface of fibers might also be a determining factor in the specificity or rate at which iron is mobilized. This did not appear to be the case for crocidolite when incubated with the chelators citrate, ADP, or EDTA in the presence of the reductant ascorbate. No change in the rate of total iron mobilization was observed.[52] EDTA, NTA, and citrate appeared to be capable of mobilizing both oxidation states of iron from crocidolite to catalyze damage to DNA in the absence of a reductant or H_2O_2.[46] In fact, 42% or 19% of the iron mobilized by NTA or citrate, respectively, under anaerobic conditions was in the Fe(II) state.[46] Since ferrozine and radical scavengers (mannitol, salicylate, or DMPO) inhibited

DNA SSB formation under these conditions, it was concluded that the Fe(II) that was being mobilized by the chelators was reacting with O_2 to produce ·OH or a similarly reactive species to damage DNA.

In conducting iron mobilization studies, not using buffers[52] and not exposing the solutions to cool-white fluorescent light[71] is important for retaining experimental integrity. Buffers such as Tris and phosphate are known to bind iron[122] and were found to inhibit mobilization of iron by ferrozine.[52] These investigators used 50 mM NaCl solutions where the pH had been carefully adjusted to 7.5 for their experiments. In addition, fluorescent light was found to potentiate the reduction of iron by the chelators citrate and NTA.[71] This can lead to overestimation of the amount of Fe(II) being mobilized and can also greatly affect other assays, such as the formation of DNA SSBs or ·OH spin trapping with DMPO. In these photocatalyzed reactions, NTA and citrate are ultimately degraded and can no longer chelate the iron. This could have an impact on longer term experiments.

A strong synergism has been found to exist between cigarette smoking and asbestos exposure in the development of bronchial carcinoma.[123,124] In investigating a chemical mechanism for this synergism, Qian and Eaton have observed that organic acids found in cigarette smoke were able to chelate and mobilize iron from asbestos.[125,126] They

identified the organic acids responsible for the mobilization as stearic and palmitic acid. Iron associated with these acids could be translocated into intact red blood cells and remained associated with the cells even after washing with desferrioxamine B.[125] These observations may explain much of the synergism of the two known carcinogens. The effect of smoking on asbestos carcinogenicity has been extensively reviewed.[123]

2. Oxygen Consumption and Hydroxyl Radical Formation

A way of comparing the reactivity of surface iron on fibers with iron mobilized into solution is to measure the amount of O_2 consumed under a variety of conditions, using an O_2-sensitive electrode. To determine whether mobilization of iron from crocidolite enhanced its reactivity, Aust and Lund incubated crocidolite fibers with the chelators citrate, NTA, or EDTA for varying periods of time to allow iron mobilization. The ability of the fiber suspension to catalyze O_2 consumption in the presence of ascorbate[97] or cysteine[127] was compared with that of the solution from which the fibers had been removed. In every case the fiber-free solution, containing the mobilized iron, had the same activity as the fiber-containing solution. Aust and co-workers[127] have also observed a strong linear correlation between the amount of iron mobilized by EDTA from crocidolite and the amount of ·OH radicals produced in

the presence of cysteine and H_2O_2, as detected by EPR using the spin trap DMPO. It should be noted that no O_2 consumption or ·OH formation was observed in the absence of chelators. Taken together, these results suggest that iron mobilized from crocidolite by citrate, NTA, or EDTA was ultimately responsible for reactions with O_2 that led to ·OH formation.

Gulumian and van Wyk have made similar observations, reporting that the ability of several types of asbestos fibers to consume O_2 in the presence of H_2O_2 and a spin trap and generate reduced oxygen species on the surface of the fiber decreased across the series of fiber samples: crocidolite > amosite > chrysotile.[128] This series also is representative of the amount of iron contained in the various fibers (see Table 1) and the intensities of the corresponding EPR spectra of the fibers in the presence of H_2O_2 and the spin trap DMPO.[128] The investigators did not specify whether iron was mobilized during the 24-h incubation time used.

2. DNA Strand Breaks

Mobilization of iron has also been shown to have a strong correlation with the amount of crocidolite-dependent DNA SSBs observed. Lund and Aust[46] investigated the ability of crocidolite to catalyze the formation of SSBs in ϕX174 RFI DNA in the presence of various chelators and reductants.

By conducting experiments in a similar manner to those described for O_2 consumption, a correlation was found between the amount of iron mobilized and the ability of crocidolite to cause the formation of DNA SSBs, suggesting that the formation of SSBs was strictly due to the mobilized rather than surface iron. They also observed an increased ability to form SSBs in the presence of a reducing agent which promoted redox cycling of the catalytically active iron.[46] Table 3 shows the percent of DNA with SSBs formed after incubation of ϕX174 RFI DNA with various mineral fibers in the presence of ascorbate with or without the chelators citrate or EDTA. Crocidolite and amosite were more active in catalyzing the formation of SSBs than either chrysotile or erionite. When a chelator was added to amosite, the result was similar to that for crocidolite, and an enhancement in the amount of SSBs was observed when a chelator facilitated mobilization. Because erionite has no native iron associated with the fibers, the addition of a chelator did not enhance the ability of the fibers to damage DNA. In a subsequent section the effect of binding of iron to fibers on the abilities of fibers to generate DNA SSBs will be discussed.

DNA SSBs have also been observed in cells after treatment with crocidolite.[105,129] Libbus *et al.*[105] utilized the method of nick translation to assess the amount of DNA SSBs induced by crocidolite. They observed a dose-dependent

Table 3. Percent DNA with Single-Strand Breaks[a] Introduced by Various Mineral Fibers

Fiber	None	Citrate	EDTA	Ref.
Amosite	26 ± 8	52[b]	96[b]	46
Chrysotile	7 ± 2	--	--	46
Crocidolite	19 ± 4	29 ± 4	87 ± 3	46
Fe(II) Loaded Crocidolite[c]	16 ± 5	40 ± 8	104[d]	157
DF Crocidolite	8 ± 4	12 ± 7	21 ± 3	157
Fe(II) Loaded DF Crocidolite[e]	20 ± 3	27 ± 7	58 ± 1	157
Erionite	ND	ND	ND	152
Fe(II) Loaded Erionite[c]	28 ± 4	19 ± 6	72[d]	152
Fe(III) Loaded Erionite[c]	16 ± 3	56 ± 5	96[d]	152

[a] The percentatage of closed-circular superhelical DNA with single-strand breaks introduced in the presence of ascorbate with or without citrate or EDTA in 30 min.
[b] Aust unpublished data.
[c] Loaded with 24 nmol Fe/mg fiber.
[d] Corrected to reflect a 30 min. incubation with DNA. Actual experiments were for 5 or 15 min.
[e] Loaded with 5.5 nmol Fe/mg fiber.
ND Not Detectable

response which reached a maximum 24 h after treatment. They also observed the same amount of SSBs in cells where no fibers were visible. The investigators proposed that oxygen radicals were responsible for this. However, this would only be possible if a transition metal, such as iron, were also available. These observations would be consistent with iron mobilization from the crocidolite. Interestingly, in these experiments, riebeckite, which is a nonfibrous, noncarcinogenic form of crocidolite, was about one-third as capable of inducing DNA SSBs as crocidolite.

Turver and Brown used identification of S_1 endonuclease sensitive sites as a method of detection of DNA damage induced by crocidolite.[129] They also observed a dose-dependent increase in the amount of DNA damage caused by crocidolite. This activity was significantly attenuated by the addition of desferrioxamine B to the cells,[129] suggesting that induction of DNA damage may have been due to iron from the fiber. The authors did not address the question of whether iron mobilization was important in these observations. However, results to be discussed on cellular mobilization of iron from crocidolite may have some bearing on the results discussed here.

4. DNA Oxidation

Mobilization of iron from crocidolite, amosite, and chrysotile has been shown to increase asbestos-dependent

formation of the oxidized nucleoside 8-OHdG.[130] The formation of this oxidized base product has been shown to cause AT to GC base pair transition[74] and has been observed under conditions producing oxidative stress conditions that lead to cancer, as previously discussed.[131] Adichi *et al.*[130] have demonstrated that the addition of H_2O_2 to a mixture of calf thymus DNA and asbestos consistently increased the level of 8-OHdG formed. The addition of the chelator EDTA to the reaction mixture including H_2O_2 increased the levels of 8-OHdG formed by 3.2-fold for Rhodesian chrysotile, 3.8-fold for Canadian chrysotile, 2.3-fold for crocidolite, and 2.7-fold for amosite, over the levels observed without EDTA. The levels of 8-OHdG were 2-fold higher for the amphibole minerals than for either variety of chrysotile in the presence of EDTA,[130] which is consistent with a greater rate of iron mobilization from crocidolite or amosite than from chrysotile. In the presence of phosphate buffer, which is known to chelate iron, chrysotile has also been shown to generate low levels of 8-OHdG from deoxyguanosine.[132]

An increase in the amount of intracellular 8-OHdG has also been reported after crocidolite treatment of cultured cells. Takeuchi *et al.*[133] observed an increase in the levels of 8-OHdG in DNA isolated from human promyelocytic leukemia cells (HL60) which were treated with crocidolite. The investigators demonstrated that the 8-OHdG observed was not due to the DNA isolation procedure, but was due to the 24-h

crocidolite treatment. The amount of 8-OHdG increased with the time of incubation. Extracellular treatment with SOD and/or catalase did not inhibit 8-OHdG formation,[133] suggesting that the formation was due to intracellular generation of a reactive oxygen species, such as the ·OH radical. Also in these studies the investigators did not determine whether iron mobilization was involved. Once again, intracellular iron mobilization from crocidolite may have some bearing on these observations.

5. Intracellular Iron Mobilization

Iron and other ions, specifically magnesium, have been observed to be leached from chrysotile asbestos *in vivo*.[107-111] The amount of iron leached *in vivo* was small and not quantified. Iron has also been reported to be mobilized from asbestos fibers by cells in culture. Chao *et al.*[134] observed that when human lung cells (A549) were treated with neutron-activated crocidolite containing ^{55}Fe, iron was mobilized intracellularly and was found associated with proteins and chelators of molecular weight <10kD. There was a linear relationship between the amount of iron in the <10kD, or low-molecular-weight pool, and the cytotoxicity, suggesting that the iron in this fraction was indeed damaging and may have been responsible for the toxic effect of crocidolite. The rate of iron mobilization in A549 cells was comparable to the rate of iron mobilization from

crocidolite by citrate *in vitro* at pH 7.5.[134] This finding suggests that iron can be removed from the fibers by intracellular chelators and that this intracellular chelator(s) may be similar in size and mobilizing ability to citrate.

The redox activity of iron in a low-molecular-weight pool in biological systems has been the subject of intense speculation, since iron has been observed chelated to low-molecular-weight chelators only in disease states, such as hemochromatosis. Citrate-chelated iron is known to cause damage to biomolecules[47,52] and has been observed in the blood of hemochromatosis patients.[40] The observation that iron is mobilized from crocidolite into a low-molecular-weight pool may prove to be very important in understanding how iron from mineral fibers becomes involved in the development of disease states. The reactivity of the iron mobilized from crocidolite may be similar to iron in other pathological conditions, since it was directly related to the cytotoxicity of the fibers.

C. Iron Binding

Mineral fibers not only can liberate iron, but also can acquire iron, under certain conditions. Because of the number and damaging potential of the reactions catalyzed by iron, additional reactive iron on fibers is likely to potentiate the dangerous nature of the fiber.

1. Ferruginous Bodies

Respirable, durable fibers are known to acquire iron on their surfaces during residence in the lung. These coated fibers are known as ferruginous bodies,[135] or asbestos bodies if the core is asbestos.[136] Crocidolite, amosite, chrysotile, and erionite are all known to form ferruginous bodies after long-term residence *in vivo*. Ferruginous bodies were first observed in 1906.[137] Not all inhaled mineral fibers form ferruginous bodies. However, the chemical properties of fibers that determine whether they become iron-coated are not known.[138]

Chronic inflammation, which occurs after inhalation of mineral fibers, is frequently accompanied by systemic changes in iron metabolism. There is a depression of serum transferrin, while the intracellular iron level in inflammatory macrophages increases. A possible explanation for this is that inflammatory macrophages appear to have a greater rate of iron uptake[139] and slower release[140] than resident macrophages. Macrophages are the primary scavengers of effete erythrocytes. This pathway has been estimated by some investigators to represent 80% of iron turnover.[141] Macrophages have been proposed to be the source of the iron in ferruginous body formation, and there may be good reason to suspect an involvement of macrophages in deposition of iron on phagocytized fibers which have a high affinity for iron.

There are two observations which indicate that macrophages accumulate iron during the inflammatory response following exposure to mineral fibers. First, the iron content in the lungs of workers chronically exposed to mineral dusts greatly increases after the influx of macrophages.[142] Second, macrophages, taken from the peritoneum of mice injected with crocidolite, accumulate iron.[143] Although the optimal time for the formation of ferruginous bodies in humans is not known, it is known that individuals vary in their ability to coat fibers. This may be due to variation in the total body burden of iron.

The formation of the asbestos bodies may begin by the deposition of monolayers of iron in high affinity sites followed by nucleation and formation of a 3-dimensional aggregate or precipitate of iron on the surface. The intracellular source of iron that has been proposed is ferritin or hemosiderin, which is oxidized ferritin from lysosomes.[138] Suzuki and Churg[144] have suggested that the ferruginous body formations are composed of iron, protein, and probably other material. It has also been demonstrated that a layer of mucopolysaccharide appears to be associated with ferruginous bodies.[145] Mature ferruginous bodies form over decades of exposure in the lung.

It has been reported that the iron on ferruginous bodies is crystalline in nature. However, the mechanism by which iron binds to ferruginous bodies or the exact source

of the iron is not well understood. Several studies have demonstrated adsorption of iron onto silicates[146,147] and binding of iron onto silanol groups on the surface of particles.[148,149] The size of ferruginous bodies varies based upon the size of the original fiber population. Koerten *et al.*[150] have observed that when crocidolite was added to cultures of macrophages, only fibers which were too large to be completely phagocytized were coated to form asbestos bodies. This may be the result of macrophage death after attempting to phagocytize fibers that are too large. Thus, formation of asbestos bodies on fibers less than 25 μm in length was not observed. After 4 weeks in culture, the large fibers were beginning to be coated, but the thickness and the segmentation of the added material became more prominent after longer times of exposure.[150] Scanning electron micrographs of an amosite core ferruginous body which was removed from a human lung at autopsy showed that the iron coating was not homogenous but formed thicker plaques at the ends and at intervals along the length of the fiber. The iron coat was approximately 0.9-1.7 μm thick.

The iron on the surface of asbestos bodies with amosite cores has been shown to be catalytically active and capable of causing the formation of SSB in ϕX174 RFI DNA[151]. This reactivity appeared to be due to iron on the ferruginous body structure, since the ability of the fibers to form SSBs was enhanced by the addition of EDTA (SSBs in 77% of DNA) or

citrate (SSBs in 21% of DNA) in the presence of a reductant and inhibited by the addition of desferrioxamine B to the reaction mixture.[151] An equal number of native amosite fibers of similar length were unable to catalyze formation of detectable amounts of DNA SSBs under the same conditions because of the low number of fibers used. Previous to these studies, it was generally accepted that the coating of fibers to form ferruginous bodies was a protective mechanism. However, because the deposited iron appears to be redox active, it may actually contribute to the catalytic potential of the fibers.[151]

2. Erionite

Several *in vitro* and *in vivo* studies have been undertaken to determine the role of iron acquisition on reactivity of mineral fibers. Eborn and Aust[152] found that native erionite was incapable of catalyzing damage to DNA *in vitro*, even in the presence of a reductant and an iron chelator, suggesting that the fiber is unable to generate reduced oxygen species. This chemical inactivity is consistent with the previous report of Zalma *et al.*,[101] who demonstrated that no ·OH-DMPO adducts were observed in the presence of erionite.

When erionite was incubated in solutions of 25 to 500 μM ferrous or ferric ions, the erionite fibers were able to remove iron from solution.[152] Erionite was capable of

binding 176 nmol Fe(II)/mg, or 239 nmol Fe(III)/mg from 500 μM ferrous or ferric chloride solutions. The investigators suggested that more ferric ions may bind through a precipitation or crystallization process,[153,154] which is consistent with what is understood about the activity of ferric ions in solution.[155] After ferrous or ferric binding, the erionite fibers acquired the ability to catalyze the formation of DNA SSBs *in vitro* in the presence of a reductant and/or chelator. The amount of SSBs was directly proportional to the amount of iron mobilized when a chelator was present.[152] Erionite, with only 24 nmol Fe(III)/mg erionite, was able to catalyze the formation of DNA SSBs in nearly 100% of the DNA in the presence of EDTA and ascorbate during a 30 min incubation. This is indeed striking since crocidolite, which contains approximately 4.8 μmol iron/mg (200 times that on erionite), was capable of catalyzing the formation of DNA SSBs in only 87% of the DNA under the same conditions[46] (Table 3).

Other investigators have also observed increases in the ability of a synthetic Y zeolite[94] or erionite[156] to catalyze the formation of ·OH after loading with Fe(II). Because of the enormous surface area of erionite, it is possible that under iron-binding conditions, large amounts of iron could be added to the fiber. The apparent discrepancy between the biochemical inactivity of erionite *in vitro* and its potent carcinogenic activity *in vivo* may be explained by

acquisition of iron in the lung after inhalation.[152]

3. Crocidolite, Amosite, and Chrysotile

The ability of crocidolite to bind iron from solution has also been examined. Hardy and Aust[157] reported that crocidolite bound 57 nmol Fe(II)/mg from $FeCl_2$ solutions at pH 7.0, which increased the iron available for mobilization by either EDTA or citrate. DF crocidolite had a diminished, but detectable ability to bind ferrous ion from solution. Binding of ferrous ion enhanced the ability of these fibers to form DNA SSBs[157] (Table 3). Treatment of iron-containing mineral fibers with chelators, such as desferrioxamine B, has been proposed as a cure for the pathological effects of the fibers. Based on these findings, it appears that this treatment is not adequate to reverse the catalytic capabilities of the fibers if they are later exposed to a source of chelatable iron.

Crocidolite and DF crocidolite were also examined for their ability to form SSBs after incubation in iron-free or iron-containing tissue culture medium.[117] Both types of fibers were more capable of inducing DNA SSBs after preincubation in an iron-containing medium than after preincubation in an identical, but iron-free medium. Fibers were also more toxic to human lung A549 cells cultured inan iron-containing medium than to cells cultured in the same medium, free of added iron. A correlation between the

crocidolite-dependent cytotoxicity in cells cultured in various media and the amount of DNA SSBs *in vitro* produced by crocidolite preincubated in the corresponding medium was reported.[117] The investigators concluded that because crocidolite was capable of acquiring iron from a complex solution with many chelators present, it is plausible that fibers may bind iron from similar low-molecular-weight chelates intracellularly. It also appears that iron bound in this manner has a biological effect, since fibers were more toxic in A549 cells after exposure to iron from the medium.

Ghio and co-workers have also been active in investigating the role of iron acquisition on the reactivity of mineral fibers. They observed that crocidolite, as well as three other silicates (silica, kaolinite, and talc) were able to bind all of Fe(III) from solutions ranging in concentrations from 1 μM to 1 mM.[158] The fibers apparently bound all of the iron from the solutions, 1 to 1000 nmol Fe(III)/mg fiber. Following binding of iron, an increase in the formation of thiobarbituric acid reactive products was observed over control fibers exposed to solutions without iron. Crocidolite showed the greatest increase in reactivity of all the fibers examined, and desferrioxamine B treatment after iron binding consistently decreased the reactivity of the fibers. Iron-treated fibers were also more active in stimulating release of leukotriene B_4, an

indicator of the inflammatory response, in rat alveolar macrophages than wetted or desferrioxamine B-treated fibers. Ghio and co-workers[158] also injected crocidolite, silica, kaolinite, or talc into the pleural cavities of rats. The fibers that were recovered 4 days later had increased amounts of chelatable iron on their surfaces. The investigators concluded from all of this work that iron was bound to fibers from both inorganic and biological sources and proposed that the iron was bound to silanol groups on the surface. The addition of iron appeared to be responsible for increased abilities to generate oxidants and was proposed to be responsible for the induction of biological activities.

In a later study, Ghio *et al.*[159] compared crocidolite, amosite, and chrysotile for their ability to bind iron from solution. Using the same iron treatment techniques with 1-mM solutions as were discussed above, crocidolite was reported to bind approximately 300 nmol Fe(III)/mg, amosite 280 nmol Fe(III)/mg, and chrysotile 175 nmol Fe(III)/mg.[159] This conflicts with the previous report by these investigators that incubation of crocidolite in 1 mM solutions resulted in 1000 nmol Fe(III)/mg.[158] This discrepancy was not discussed, but may reflect the difficulties that can be encountered in handling $FeCl_3$ solutions or may be due to the manner in which binding was being assessed. In any case, the amount of DNA SSBs

increased after iron binding by approximately 19% for crocidolite, 13% for amosite, and 4% for chrysotile. A similar increase in the generation of oxidants, as measured by the thiobarbituric acid reactive products assay, was observed. After intrapleural injection of fiber suspensions, 240, 135, or 25 nmol Fe/mg were observed on crocidolite, amosite, or chrysotile, respectively.[159] It is likely that additional iron from intracellular sources would demonstrate the same types of effects on induction of DNA SSBs as the iron from inorganic sources, but this was not investigated. As before, the investigators proposed that the silanol groups on the surface of a mineral fiber may be responsible for binding cations from solution. The investigators proposed that one potential method for determining whether the fibers made to replace asbestos will be dangerous may be to analyze for the concentration of silanol groups on the surface.[159] Although this may have some merit in identifying potentially hazardous materials, it is by no means definitive, since erionite, the most carcinogenic mineral fiber, has approximately 1000-fold fewer silanol groups, but binds as much or more iron as crocidolite (see Tables 1 & 3).

VI. Fiber Inactivation

The unique blend of physical properties possessed by asbestos fibers, coupled with the high demand for such

materials in a variety of applications, has stimulated several investigators from around the world to modify fibers in hopes of rendering them biologically inactive.

A. Ferric Oxide Coating

Gulumian and co-workers[160,161] have been active in studying modifications of crocidolite which may render the fibers less biologically active. These investigators used a method of coating asbestos fibers with ferric oxide (Fe_2O_3), which was developed by Flowers.[162] The effects of this treatment on the bulk and surface of crocidolite fibers were studied in detail.[161] Mössbauer studies revealed that ferrous ions were oxidized to ferric ions in specific sites in the mineral fiber, and that no changes in the structure occurred during the treatment. Furthermore, the surface concentration of ferric ions increased. The chemical formula of the surface complex was interpreted to be $Fe(H_2O)_6$. This treatment rendered the fibers less capable of releasing ferrous ions to ferrozine and less capable of catalyzing the formation of $\cdot OH$ in the presence of H_2O_2, detected by EPR spin trapping with DMPO.[160] Since ferrous ions were still available for mobilization and because the detoxified fibers remained capable of generating reduced oxygen species, it appears that this type of bulk and surface modification was not sufficient to completely inactivate the fibers, rendering them safe for human

exposure.

In an interesting reverse study, the activation of crocidolite fibers was conducted using H_2 gas as a reductant for crocidolite iron.[163] This treatment increased the amount of Fe(II) by reduction of Fe(III). The activated fibers released more Fe(II) to ferrozine chelation and were able to generate more ·OH[163] than unactivated fibers. The converted Fe(II) was stable for at least 3 months. While the reducing conditions used to activate these fibers were much different than those that would be encountered in the cell, these studies still point out that iron on fibers might be reactivated by conversion from ferric to ferrous in the presence of cellular reductants. This raises serious questions about the use of iron to prevent the pathological effects of fibers.

B. Polymer Coating

Brown *et al.*[164] were successful in binding C_8 or C_{18} polymers to the surface of amosite by refluxing the fibers in octyldimethylchlorosilane (C_8) or octadecyldimethylchlorosilane (C_{18}) in toluene for 6 h. The modified fibers were less able to associate with cultured V79 cells and were less toxic to these cells than control fibers. The investigators proposed that fibers modified in this way might produce less damage *in vivo*. However, when the same fibers were intrapleurally injected into rats, the

native, C_8-modified, and C_{18}-modified fibers were all carcinogenic.[164] Although the C_{18}-modified fibers were less carcinogenic than the native fibers, the investigators concluded that this method of inactivation was not viable because it did not render the fibers completely safe for human exposure.

C. Chelation Treatment

Chao and Aust[113] explored the potential of long-term mobilization as a method of inactivating fibers. After removal of only 4.5% or 1.2% of the total iron from crocidolite or amosite, respectively, the fibers were no longer capable of causing DNA SSBs in the presence of ascorbate and EDTA in a 30-min incubation. This finding suggests that only a small percentage of the iron on crocidolite and amosite is available for chelation and mobilization away from the fiber by one specific chelator in a 15-day treatment. Nevertheless, subsequent treatment of the same fibers with the same or another chelator resulted in further mobilization of iron from the fibers.[113] The investigators concluded that although the removal of iron decreased the activity of the fibers, it would not completely inhibit the activity in a biological system with longer exposure times. Perhaps an even greater hazard would come if the fibers from which iron was removed subsequently bound iron, as was described previously.[157] This would

reconstitute their damaging capabilities.

In summary, attempts to modify asbestos to render the fibers safe for use and human exposure have been rather unsuccessful. Several of the modifications have lowered the ability of the modified fibers to catalyze reactions *in vitro*, specifically generation of .OH, formation of DNA SSBs, or association with cells. None of the modifications attempted, to date, have been capable of completely inhibiting catalytic reactivity. The findings of Brown and co-workers[164] suggest that even when a demonstrable decrease in an *in vitro* parameter is observed after modification of fibers, a concomitant decrease in carcinogenicity may not be observed. Due to the durability of asbestos fibers in the lung, it is likely that inactivation of fibers by modification might change their properties, rendering them less useful.

VII. Physiological Effects

It is difficult to know which of the many carcinogenicity tests *in vitro* and in animals are the most important in predicting human carcinogenicity. Johnson[165] has suggested that animal inhalation models are relevant for identifying hazardous fibrous materials in the absence of epidemiological data, since use of intrapleural or intraperitoneal installations can give false positive results. However, when two parameters correlate well, it

suggests that perhaps what is observed in one is reflected in the other. Maples and Johnson[166] demonstrated a correlation between the tumor incidence after intrapleural administration of erionite, crocidolite, amosite, or chrysotile in rats with the ability of these fibers to generate ·OH *in vitro* in the presence of H_2O_2 (r^2=0.896). There also appeared to be a strong correlation between the mortality rate for mesothelioma in humans and the ability of these fibers to produce ·OH (r^2=0.990). No correlation was observed between the ability of fibers to cause tumors after intraperitoneal administration in rats and their ability to generate ·OH.[166] This is in agreement with previous reports by Carthew *et al.*,[15] who observed a similar correlation between the carcinogenicities of mineral fibers in humans and the relative carcinogenicities of mineral fibers administered intrapleurally in rats, but not with mineral fibers administered intraperitoneally in rats. They found that, as in human exposure, intrapleural administration of erionite was much more carcinogenic than crocidolite, which was more carcinogenic than chrysotile.[15]

The abilities of crocidolite, amosite, and chrysotile to generate ·OH as in the Maples and Johnson study[166] seem to generally correlate with both the iron content and the amount of iron mobilized from these fibers, as demonstrated by other investigators.[46] However, the generation of ·OH by erionite observed by Maples and Johnson[166] is not easily

explained, since erionite contains little or no iron. Other investigators have consistently found erionite unable to generate ·OH,[152] even after grinding.[156] One possible explanation for the observed generation of ·OH by erionite may be that a catalytic amount of iron was introduced to the fibers as a contaminant from solutions used in these studies. Even a small amount of iron bound to zeolites is very reactive, as previously discussed.[94,154] This study, combined with information from other studies,[52] suggests that both the amount of iron on the fiber and the amount of iron which can be mobilized from the fiber may be related to the rate of human mortality from mesothelioma through the iron-catalyzed generation of reduced oxygen species, specifically the ·OH.

A. Participation of Iron

The role of iron has been extensively studied using desferrioxamine B in a number of different physiological reactions. When fibers were pretreated with desferrioxamine B, the fibers were less able to form malonaldehyde-like products[118] or to cause lipid peroxidation *in vitro*.[167,168] The cytotoxicity of desferrioxamine B-pretreated crocidolite was lower than untreated crocidolite in human lung cells,[114,117] macrophages,[30,115,116,119] red blood cells,[118] fibroblasts,[119] and endothelial cells.[120] Kamp *et al.*[169] treated human pulmonary epithelial cells with amosite asbestos in the presence of

polymorphonuclear leukocytes and saw a decrease in cytotoxicity with the addition of desferrioxamine B. These studies utilizing desferrioxamine B seem to indicate that a number of varied reactions associated with exposure of cultured cells to asbestos fibers are catalyzed by iron.

Active oxygen species generated by iron on amosite have been reported to be involved in the uptake of the fibers by rat tracheal epithelial cells.[170] Evidence for this hypothesis is that increasing concentrations of desferrioxamine B were progressively more capable of preventing fiber uptake at concentrations of 10 μM to 1 mM. The anti-oxidant scavenger enzymes superoxide dismutase and catalase were also increasingly effective at inhibiting fiber penetration as the concentration increased from 325 to 1300 U/mL catalase or 150-1200 U/mL superoxide dismutase in 1 or 3 days. The inactive enzymes were not capable of inhibiting fiber uptake in the same manner as the active enzyme, suggesting that the normal activity of the enzymes was required.[170] The authors did not conclude which kind of cellular damage was required for amosite uptake, but suggested that since catalase, superoxide dismutase, and desferrioxamine B were all able to independently inhibit fiber uptake, H_2O_2, $O_2^{\cdot-}$, and iron are all involved in the mechanism of uptake.

B. Antioxidant Proteins

Reactions catalyzed by iron are known to generate strong oxidants. When a cell experiences conditions where the levels of these species are elevated, the condition is commonly referred to as "oxidative stress." Many of the complex physiological reactions to oxidative stress have previously been reviewed[123] and will not be the focus here. A few of the most recent and important findings regarding asbestos-induced oxidative stress will be included because they suggest that oxygen radicals are being generated in the lungs of asbestos-exposed animals or in treated cells.

The synthesis of a variety of proteins, e.g. glutathione, glutathione peroxidase, catalase, and SOD, can be increased in response to oxidative stress conditions. Glutathione is thought to be an important intracellular antioxidant protein. Glutathione peroxidase eliminates organic peroxides and H_2O_2 while catalase catalyzes the decomposition of H_2O_2 to H_2O and O_2. Superoxide dismutase accelerates the dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2.

Asbestos has been shown to induce synthesis of several proteins which play pivotal roles in the cellular defense against oxygen radicals.[171,172] Janssen *et al.*[172] observed that after inhalation of asbestos by rats, steady-state levels of mRNA for two forms of superoxide dismutase and glutathione peroxidase were elevated relative to untreated controls. The overall enzyme activities of catalase, glutathione peroxidase, and both forms of superoxide dismutase also

increased in the exposed lung cells after asbestos exposure.[172] Holley *et al.*[171] have also shown that treatment with crocidolite increases the levels of mRNA for Mn superoxide dismutase. A dose-dependent increase in glutathione has been observed after administration of crocidolite to pulmonary alveolar macrophages *in vitro*.[173] The induction of antioxidant proteins suggests that the same types of cellular signals that are associated with oxidative stress, e.g. generation of reactive oxygen species, are associated with the reactions catalyzed by asbestos.

VIII. Mutations and Cancer

Carcinogenesis appears to be a multistep process that has been divided into two stages, initiation and promotion. The initiation phase is believed to be the introduction of a heritable genetic change (mutation) resulting from carcinogen-induced DNA damage. With the discovery of oncogenes, which are activated by mutations to cause cancer, and tumor suppressor genes, which are inactivated by mutations leading to cancer, some of the genetic targets for this damage have been identified. Initiation is followed by the promotion phase in which the initiated cell proliferates, undergoing further changes that result in the malignant phenotype. By studying the initiation phase of cancer, a variety of assays have been developed to assess the mutagenicity of carcinogens. Among these is the well

known Ames assay with bacteria. Surprisingly, asbestos is relatively inactive in almost all mutation assays. This may not be so difficult to understand if iron is responsible for the DNA damage induced by asbestos.

It appears from work with cultured cells that asbestos must be phagocytized in order to exert its toxic effects.[174] For mammalian cells, phagocytosis of high iron content asbestos fibers would represent an uncontrolled entry of iron into the cells. Since phagocytosis is not possible for bacteria, the fibers would never enter the cells where they might be able to catalyze the formation of reactive oxygen species. Thus, no mutations would be observed when the bacteria were incubated with the fibers. But fibers are phagocytized by mammalian cells. Why then are the fibers not causing mutations? The answer appears to lie in the type of DNA damage induced by the fibers.

It is well-known that asbestos causes chromosome aberrations, both structural and numerical, in mammalian cells.[175] This chromosomal damage may be the basis for asbestos-induced transformation of Syrian hamster embryo cells.[176] While it has been proposed that chromosome aberrations are the result of direct fiber interaction with DNA or spindle proteins,[177] it may be that some, or perhaps all, of the damage is the result of iron-catalyzed oxygen radical attack on DNA, which has been shown to occur in cultured cells.[129] This is very consistent with what is

observed for ionizing radiation which generates ·OH. Interestingly, it is also somewhat difficult to observe the induction of mutations by ionizing radiation. Special bacterial strains were developed to be used in the Ames assay for the purpose of detecting oxygen radical damage.[178] Until recently, no such strains were available in mammalian cells. With the development of the A_L hamster-human hybrid cell line, Hei *et al.*[179] have been able to observe mutations by ionizing radiation and by asbestos at the a_1 locus of human chromosome 11 in the A_L cell line. Hei *et al.*[180] also observed that reactive oxygen species induce mostly deletion mutations in mammalian cells. This work suggests that asbestos damages DNA to cause deletion mutations via iron-catalyzed generation of oxygen radicals. The induction of deletion mutations can be very toxic to the affected cell, and this probably explains why asbestos-induced mutations have been rarely observed except in the A_L hybrid cell line.

Current understanding of the initiation and promotion events leading to cancer reveals that the expression of oncogenes and tumor suppressor genes is important in regulation of cellular function. The protooncogenes c-*fos* and c-*jun* encode a family of AP-1 transcription factors that form homodimeric and heterodimeric protein complexes. These AP-1 transcription factors bind to specific regulatory sequences in DNA to control the transition from G_1 to S phase in the cell cycle. In rat pleural mesothelial cells

and hamster tracheal epithelial cells, c-*fos* and c-*jun* have been shown to be persistently induced by treatment with asbestos.[181] The mRNA synthesis for these oncogenes was induced in a dose-dependent manner and persisted for at least 24 h. Crocidolite was more active than chrysotile in inducing mRNA synthesis for these two genes in mesothelial cells. This emphasizes the relevance of this work, since crocidolite is known to induce mesothelioma more easily than chrysotile. In the tracheal epithelial cells, c-*jun* was induced by crocidolite or chrysotile, but c-*fos* was not. The protein transcription factor AP-1 was also persistently observed. Treatment of either cell line with polystyrene beads or riebeckite did not induce c-*fos* or c-*jun*, suggesting that the response was specific to fibrous asbestos minerals and was not stimulated by the presence of a solid body alone. Induction of these proteins could lead to chronic stimulation of cell proliferation.[77] Currently, this study provides some of the molecular understanding of the consequences of asbestos exposure that may cause cancer.

IX. Conclusions

Findings compiled in this review support the view that intrinsic or acquired iron is responsible for the biochemical properties of some of the most carcinogenic minerals. Many studies have shown a direct relationship between the biochemical reactivity of fibers and the iron

content of those fibers. Iron acquisition was the key factor in converting erionite from a biochemically unreactive form to a highly reactive form. This may explain the apparent discrepancy between the *in vitro* inactivity of erionite and its highly carcinogenic activity *in vivo*. The mobilization and binding of iron appear to be key factors in the regulation of reactivity of fibers both *in vitro* and *in vivo*. Several of the physiological responses to asbestos have been decreased by the use of desferrioxamine B, suggesting that these intracellular responses to asbestos are mediated or generated by iron. Ultimately, the fundamental differences between durable mineral fibers in their abilities to bind and later release iron may explain why some are more toxic and carcinogenic than others.

Significant advances have been made in the field of mineral surface science due to the development of X-ray photoelectron spectroscopy, Auger electron spectroscopy combined with scanning electron microscopy, and scanning force microscopy. Thus, the surface requirements for iron binding and effects of mobilization will undoubtedly be investigated and compared among the carcinogenic fibers to determine what factors are necessary for reactivity of the fibers. Studies such as these will aid in the design of man-made mineral fiber replacements for asbestos that are safe for human exposure.

Many investigators have studied the ability of asbestos

to catalyze damage through the iron-mediated generation of reactive oxygen species. This appears to be one of the most important chemical reactivities catalyzed by asbestos fibers in relation to the long-term effect of cancer. Work summarized here showed a strong correlation between the ability of fibers to generate reduced oxygen species and the mortality rate from mesothelioma in humans. Since these reactions require iron to be very near the target for damage, mobilization of iron from the fibers may be a key step, and studies were discussed which provide evidence that iron can be mobilized *in vitro* and in cultured cells.

Intermediate reactive factors produced by iron-catalyzed reactions, which have not been extensively studied with relation to mineral fibers, are the reactive aldehydes produced from lipid peroxidation. These species are longer lived and more mobile than some of the oxygen radical species discussed and may be involved in mediating biological response. Another reactive oxygen species which may be involved in mediating biological response is nitric oxide. Nitric oxide is released from activated macrophages and may be released from other types of cells under conditions of oxidative stress. Nitric oxide can participate in many reactions with iron and with reduced oxygen species produced by iron-catalyzed reactions. Thus, this will likely be a research area that will be developed in the coming years.

Future studies will address the exact interaction of iron and other components of mineral fibers with various oncogenes and tumor suppressor genes. Early studies on the protooncogenes c-*fos* and c-*jun* suggest that indeed asbestos-induced carcinogenicity may be regulated by a complex pathway of signals which are influenced and perhaps even directed by asbestos. Studies like those on induction of c-*fos* and c-*jun* are critical to understanding cellular response. As the interaction between biological molecules and asbestos fibers becomes more clear, possible treatments for people who have already been exposed to asbestos and will suffer from asbestos-induced cancer may be feasible.

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CHAPTER 2

THE EFFECT OF IRON BINDING ON THE ABILITY OF CROCIDOLITE AND SILICON CARBIDE TO CATALYZE DNA SINGLE-STRAND BREAKS[1]

Abstract

Crocidolite or crocidolite pretreated with desferrioxamine B (DF crocidolite) or one of three varieties of silicon carbide whiskers (SiCWs) was exposed to ferrous chloride solutions to determine whether iron could be bound from solution. Native crocidolite was capable of binding up to 57 nmol Fe^{+2}/mg fiber in 60 min while the DF crocidolite was capable of binding only 5.5 nmol Fe^{+2}/mg fiber. The rate of iron binding for the first 5 min of exposure was independent of the concentration of iron in the solution, suggesting that there is a group of rapidly saturable sites, approximately 1.5×10^{18} binding sites/m^2 crocidolite surface, that are responsible for the immediate binding. This process is followed by a slower binding phase, likely to occur at other sites. SiCWs were capable of binding from 2.9 to 29.0 nmol Fe^{+2}/mg fiber. Crocidolite, DF crocidolite, and with various amounts of iron bound were assayed for their abilities to catalyze the formation of DNA single-strand breaks (SSBs) in ϕX174 RFI DNA. Native crocidolite

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[1] Coauthored by Jeanne A. Hardy and Dr. Ann E. Aust

with additional iron bound did not significantly change in its ability to cause DNA SSBs; however, DF crocidolite with the addition of iron had a significantly increased ability to form DNA SSBs. DF crocidolite with an additional 0, 3.0, or 5.5 nmol Fe^{+2}/mg catalyzed the formation of DNA SSBs in 21, 42, or 51% of the DNA, respectively, in the presence of EDTA and ascorbate. More iron was mobilized from iron-treated crocidolite than from untreated crocidolite in 4 or 24 hours. Fibers were also incubated in tissue culture medium with or without iron salts. The fibers incubated in the iron-containing medium had an increased ability to form DNA SSBs, under certain conditions. These results suggest that fibers such as crocidolite may be capable of binding iron from intracellular sources. This additional iron may be as reactive as the intrinsic iron and may increase the reactive lifetime of the fiber.

Introduction

It has been well documented that exposure to crocidolite asbestos increases the risk for mesothelioma of the pleura or peritoneum as well as carcinoma of the lungs, esophagus or stomach (reviewed in 1,2). Results from many studies have indicated that the dimensions of asbestos fibers may be linked to their biological effects after inhalation (3). This hypothesis, however, does not explain the biochemical reactivity of asbestos. The molecular

mechanism for crocidolite carcinogenesis, as well as for fiber carcinogenesis in general, remains obscure.

Recent studies have indicated that the carcinogenicity of crocidolite fibers may be related to the iron content of the fiber. Iron appears to be involved in several conditions leading to cancer. Persons who suffer from hereditary hemochromatosis (4,5) and porphyria cutanea tarda (6,7), which are iron overload conditions, are at greater risk for developing liver cancer. There is also a correlation between high body stores of iron and an increased risk for developing cancers of all types (8-10).

Crocidolite is a member of the amphibole class of minerals and contains 27% iron by weight (11). Crocidolite is composed of layers of infinite, one-dimensional chains of silicon-oxygen tetrahedra. Stacked silicate chains are stabilized by Van der Waals forces between the chains to form the mineral structure. The cations Mg^{+2}, Na^{+1}, Fe^{+2}, and Fe^{+3} are sandwiched between the silicates, further stabilizing the three-dimensional structure (12, 13). Minor differences in the overall composition of crocidolite have been reported, depending on the location of mining and on the milling process (14).

Silicon carbide whiskers (SiCWs) are the by-products of manufacture of materials such as computer chips, which are composed of silicon carbide. Additional metals are sometimes trapped as inclusions in the whiskers. Workers in certain

industries are commonly exposed to these particles, which have similar size and respirability to asbestos. The biological effects of these whiskers are not fully understood.

The latency for development of mesothelioma and bronchogenic carcinoma following asbestos exposure is 20 to 40 years; however, people have only been extensively exposed to SiCWs for the past 20 years, so comprehensive epidemiological data on the risks of exposure to SiCWs have not been compiled. Certain varieties of SiCWs have been shown to be carcinogenic in animals and appear to induce some of the same acute effects as asbestos does. Unlike asbestos usage, the use of silicon carbide is not rigorously controlled. Therefore, if silicon carbide is as carcinogenic as asbestos, a new epidemic of workers exhibiting cases of fiber-induced cancer could be only a few decades away. This study examines some of the *in vitro* effects of SiCWs, with the prospect that other, more comprehensive and definitive studies will follow, so that the question as to whether silicon carbide is a respiratory threat or not can be answered.

Crocidolite has been shown to catalyze the same types of reactions as iron, namely generation of reactive oxygen species (15-17), oxidative damage to DNA (18,19), and lipid peroxidation (20). Lund and Aust have shown that mobilization of iron from asbestos fibers increased the rate

at which SSBs were introduced in DNA (21) and increased crocidolite-dependent oxygen consumption (22). Iron from asbestos has also been reported to be mobilized from crocidolite intracellularly. The amount of iron mobilized in human lung carcinoma cells correlated with crocidolite-dependent cytotoxicity (23). Iron which is mobilized from fibers, or which is coordinated in accessible sites on the surface of a fiber, may be capable of causing damage to biomolecules through the modified iron-catalyzed Haber-Weiss mechanism, shown below (24).

$$\text{Reductant}^{(n)} + \text{Fe(III)} \rightarrow \text{Reductant}^{(n+1)} + \text{Fe(II)} \quad (1)$$

$$\text{Fe(II)} + O_2 \rightarrow \text{Fe(III)} + O_2^{\cdot -} \quad (2)$$

$$HO_2^{\cdot} + O_2^{\cdot -} + H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

$$\text{Fe(II)} + H_2O_2 \rightarrow \text{Fe(III)} + OH^- + OH^{\cdot} \text{ (Fenton Reaction)} \quad (4)$$

Ascorbate and other cellular reductants can potentiate the reactivity of iron. Hydroxyl radicals are known to react with DNA, hydroxylating nucleotide bases and introducing strand breaks (25,26).

When iron is removed from crocidolite by chelation and mobilization, a detoxified form of the mineral fiber is obtained. Treatment of crocidoite with desferrioxamine B for 90 days removed 8% of the iron from the fiber. Crocidolite treated with desferrioxamine B for varying periods of time has been shown to have lower toxicity than native crocidolite in Syrian hamster embryo cells (27), RL-82 cells (28), or mouse peritoneal macrophages and macrophage-like

$P388D_1$ cells (29). Iron can be mobilized from crocidolite by cultured human lung cells (23). Thus, DF crocidolite may serve as a model for fibers which have existed under similar conditions in the body.

In addition to having a high natural iron content, fibers which persist in the lung for several decades, such as crocidolite (30), acquire iron on their surfaces. Depostition of large amounts of iron on the surface of a particle constitutes formation of a mature "ferruginous body," which can be detected even by light microscopy (31-33). Ferruginous bodies were first observed in humans in 1906 (34) and crocidolite is amongst the group of fibrous and nonfibrous particles known to form ferruginous bodies (35-37). Scanning electron micrographs of ferruginous bodies extracted from an autopsied human lung showed that the iron coat was approximately 0.9-1.7 μm thick and was not evenly distributed upon the fiber, but formed thicker, dumbbell-shaped plaques on the ends, with knobs at intervals along the length of fiber (31,38). The formation of ferruginous bodies may begin by the deposition of monolayers of iron in high affinity sites followed by nucleation and formation of an aggregate or precipitate of iron on the surface. Coordination of iron by several crystalline silicates *in vitro* has been demonstrated (39,40), and our results with erionite are consistent with these reports (41). Ghio *et al.* have shown that crocidolite fibers injected intrapleurally

in rat lungs and left for 4 days appeared to acquire iron (39). They did not determine whether this iron acquisition increased the redox capabilities of the fibers. However, results from our laboratory showed that the iron deposited on mature ferruginous bodies removed from human lungs at autopsy was redox active and was capable of catalyzing the formation of DNA SSBs *in vitro* (42). Since the fibers studies by Ghio *et al.* (39) resided in rats for only 4 days, their observations suggest that iron deposition begins very soon after inhalation of fiber, long before mature ferruginous bodies can be observed. This may represent an early monolayer deposition of iron that may contribute significantly to the biochemical and pathological activities of these fibers.

The present study was undertaken to explore the capabilities of native or DF crocidolite or any of three varieties of SiCWs to bind iron from solution. Ferrous chloride solutions were examined because it has been suggested that the majority of intracellular iron exists in the ferrous state (43,44). In addition, ferric ions in solutions pH 3-10 are known to undergo rapid hydrolysis (45), forming polymers which are likely to associate with fibers as precipitates. These ferric-containing polymers are not likely to be present *in vivo*. Native crocidolite bound iron from solution more readily than DF crocidolite, but did not show significant changes in its ability to form DNA

SSBs, while iron-treated DF crocidolite did. Both native and DF crocidolite, incubated in tissue culture medium containing iron, showed an increased ability to catalyze the formation of DNA SSBs, under certain conditions. These results suggest that iron can be bound to crocidolite and that additional iron on the fibers increases their ability to catalyze damage in biological systems.

Materials and methods

Asbestos and reagents

Crocidolite asbestos was obtained from Dr. Richard Griesemer, NIEHS/NTP (Research Triangle Park, NC), and contained 27% iron by weight (11). Silicon carbide whiskers were a gift from Dr. Neils Johnson (Loveless Inhalation Institute, Albuquerque, NM). The iron chelator, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p-sulphonic acid (ferrozine) and ferrous chloride tetrahydrate were obtained from Aldrich Chemical Co. (Milwaukee, WI). Desferoxamine mesylate USP (desferrioxamine B, N-[5-[3-[5-(aminopentyl)hydroxy-acetamide)pentyl]carbamoyl] propionohydroxamic acid monomethaesulfonate) was obtained from CIBA (Summit, NJ). Ethidium bromide and the sodium salt of L-ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Ammonium acetate, trichloroacetic acid, and NaOH were obtained from EM Science (Cherry Hill, NJ). Nitrogen gas was obtained from Liquid Air (Salt Lake City, UT). Chelex 100 was obtained from Bio-Rad Laboratories

(Richmond, CA). Sodium chloride, the disodium salt of EDTA, ferrous sulfate hexahydrate, and sodium citrate dihydrate were obtained from Mallinckrodt Inc. (Paris, KY). A custom preparation of Ham's F-12 tissue culture medium, free of added iron salts, was obtained from Gibco BRL (Grand Island, NY). Agarose was obtained from FMC BioProducts (Rockland, ME). Closed-circular, superhelical ϕX174 RFI DNA was obtained from New England Biolabs (Beverly, MA). All remaining reagents were purchased in the highest purity possible.

All NaCl solutions were Chelex-treated before use in the following experiments. DNA was removed from the shipping buffer by ethanol precipitation, as described in Maniatis *et al.* (46), before redissolving in 50 mM NaCl, pH 7.5. Ascorbate solutions were prepared immediately prior to use. All experiments were performed in darkness under red light to prevent the photochemical reduction of iron (47).

Preparation of crocidolite and SiCWs

Native crocidolite and the three varieties of SiCWs were weighed and suspended in solution immediately prior to use. Soaked crocidolite was prepared as a control for DF crocidolite by incubation at 1 mg/mL in sodium bicarbonate solution (36 mM) for 90 days. Assays for iron in the supernatant indicated that no iron was leached from the fibers during this period. DF crocidolite was prepared by

chelation treatment with desferrioxamine B (1 mM) for 90 days, as previously described (48). Assays of the supernatant after treatment were performed by USU Analytical Laboratories (Logan, UT) by a Thermojarrell Ash ICAP 9000 inductively coupled plasma emission spectrometer (ICP). ICP analysis of the supernatant of the desferrioxamine B treatment revealed that 2.66±1 ppm silicon was dissolved in the supernatant. The general formula of crocidolite is $[Na_2Fe^{(III)}_2Fe^{(II)}_3Si_8O_{22}(OH)_2]$. The amount of silicon in crocidolite comprises 30% of the total weight. The total amount of crocidolite lost to dissolution during incubation was calculated based on the amount of silicon that dissolved. These calculations demonstrated that only 1% of the total mass of crocidolite was dissolved during the treatment.

Iron binding to crocidolite and SiCWs

Ferrous chloride solutions were prepared by dissolution of $FeCl_2$ into deionized water which had been purged of dissolved oxygen by bubbling with nitrogen gas for 15 min. Solutions were maintained under anaerobic conditions for the duration of the binding assay to reduce the risk of ferrous oxidation and subsequent formation of ferric hydroxides and oxyhydroxides. Crocidolite, soaked crocidolite, DF crocidolite, or SiCWs were suspended and incubated without shaking at 1 mg/mL in $FeCl_2$ (25, 50, 100 μM) in the dark. At 5, 15, 30, 60, or 120 min, fiber suspensions were

centrifuged at 11,000 x g in a Fisher Scientific 59A microcentrifuge for 3 min to remove the fibers from supernatant. The supernatant was assayed spectrophotometrically for iron using total iron assay with ferrozine as previously described (49). The amount of iron bound to the fibers was calculated as the iron concentration in the supernatant at the time of suspension minus the iron concentration in the supernatant after the indicated time period. Surface coverage was calculated based upon the molar amount of iron bound per milligram crocidolite, knowing the average surface area of the NIEHS sample of crocidolite to be 10.1 m^2/g (11). Controls showed that no iron adsorbed to plastic Eppendorf tubes used for these studies. No iron leached from the crocidolite suspended in deoxygenated water or dilute HCl solutions of the same pH as the ferrous chloride solutions, in 1 hour.

Determination of iron concentrations

The concentration of ferrous iron in solutions before treatment of fibers was determined by a modification of the total iron assay. Ferrous-containing solutions were added directly to ferrozine (70 mM) in the absence of a reductant, or any other reagents. The colored ferrous-ferrozine complex was assayed spectrophotometrically as in the total iron assay at 562 nm on a Shimadzu UV160U UV-visible recording spectrophotometer. When assaying for total iron in the

solution, the total iron assay was performed exactly as described (49). The amount of ferrous in solution after loading was always equal to the total amount of iron in the solution, indicating that solution iron was not oxidized to the ferric state during the incubation period.

Identification and quantification of ions in solution

Ions released into the supernatant during ferrous loading were determined by USU Analytical Laboratories (Logan, UT) by a Thermojarrell Ash ICAP 9000 ICP. Iron remaining in solution following the binding incubation as determined by ICP agreed with the results of the total iron assay.

Induction of DNA single-strand breaks by crocidolite and SiCWs

After crocidolite or DF crocidolite or SiCWs had been incubated in $FeCl_2$ solutions for 60 min, the fibers were washed five times to remove loosely associated iron. Washing was performed by suspension in nitrogen-purged ddH_2O and centrifugation at 11,000 x g for 3 min. Examination of the washing supernatant showed that no ferrous ions were removed from the fibers during the washing procedure. Fibers were then resuspended in NaCl (50 mM, pH 7.5), adjusted to pH 7.5, and assayed for their ability to catalyze the formation of SSBs in ϕX174 RFI DNA as described previously (21). Washed fibers (20 μg) were incubated with ϕX174 RFI DNA

(0.25 μg). All SSB assays reported here were performed in the presence of 1 mM ascorbate, with or without 1 mM citrate or 1 mM EDTA. When native and soaked crocidolite were assayed for their ability to induce DNA SSBs in the presence of EDTA, the fibers were incubated with DNA for 15 min in the dark. This length of incubation prevented introduction of DNA SSBs in all of the DNA. All other incubations were for 30 min in the dark. After the incubation, DNA with SSBs was separated from closed-circular, superhelical DNA (RFI), using agarose gel electrophoresis. The amount of DNA with SSBs was quantified using integrated scanning densitometry as previously described (21) and expressed as percent of untreated control.

Mobilization of iron from crocidolite

Crocidolite treated with iron as previously described was suspended 1 mg/mL in 1 mM citrate or EDTA solutions. The pH of the solutions was adjusted to 7.5 every 15 minutes for the first 60 min and every hour for the first 8 hours. At 4 and 24 hours fibers were centrifuged at 11,000 x g in a Fisher Scientific 59A microcentrifuge for 3 min. The supernatant was assayed by the total iron assay. The amount of iron mobilized in the indicated time periods is expressed as nmol Fe/mg crocidolite.

Iron binding from tissue culture medium

Two types of tissue culture media were used for these studies, Ham's F-12 containing no Fe_2SO_4 (F-12), or F-12 containing 6μM Fe_2SO_4 (Fe F-12). The iron-containing F-12 was prepared by adding $Fe_2SO_4 \cdot 6H_20$ to the dry medium before dissolution. Crocidoite or DF crocidolite was suspended (10 μg/mL) in F-12 or Fe F-12 for 1 hour in the dark with shaking. The fibers were removed from the dilute suspension by centrifugation at 160,000 x g with a Beckman SW 40 Ti rotor. The recovered fibers were resuspended in 50 mM NaCl, pH 7.5, at 1 mg/mL and washed five times as described in **Iron binding to crocidolite and SiCWs**, and resuspended in NaCl (50 mM, pH 7.5) for use in the single-strand break assay, as described previously.

Results

Ability of crocidolite and SiCWs to bind iron

Initial studies were performed using $FeCl_3$ solutions. Assays of the starting $FeCl_3$ solutions using the total iron assay with ferrozine indicated that iron was being removed from the soluion. Atomic absorption analysis of the same solutions showed that the iron was still present, but was not available for chelation by ferrozine (unpublished data). This suggested that the ions were beginning to associate into ferric hydroxide polymers, which have been reported to

form immediately in pH 3-10 aqueous solutions (45). These ferric hydroxides and oxyhydroxides are likely to precipitate onto the surface of the fibers, but are unlikely to occur intracellularly. It has been suggested that most intracellular iron may exist in the ferrous state, since the cell maintains an internal reducing environment, and because most iron-containing and storage proteins require iron to exist in the reduced state for incorporation (43,44). Therefore, studies using ferrous iron were designed to avoid possible artifacts due to the formation of ferric ion polymers and because they may be more relevant to intracellular conditions.

As shown in Fig. 6, crocidolite, soaked crocidolite, and DF crocidolite were capable of acquiring iron from ferrous chloride solution. The soaked fibers demonstrated nearly identical capacities to acquire iron from solution as the native fibers. When the native or soaked crocidolite was incubated in anaerobic 25 or 50 μM $FeCl_2$, all iron was removed from solution by the crocidolite so that 25 and 50 nmol Fe^{+2}/mg crocidolite were bound after 1-hour incubations. Upon incubation in 100 μM $FeCl_2$, approximately 57% of the iron bound to the native or soaked crocidolite. DF crocidolite had a significantly diminished ability to bind iron from $FeCl_2$ solution in 1 hour. Incubation of the DF crocidolite in 25, 50 or 100 μM $FeCl_2$ for 1 hour resulted in binding only 3.0±0.6, 4.4±0.9, or 5.5±0.8 nmol Fe^{+2}/mg DF

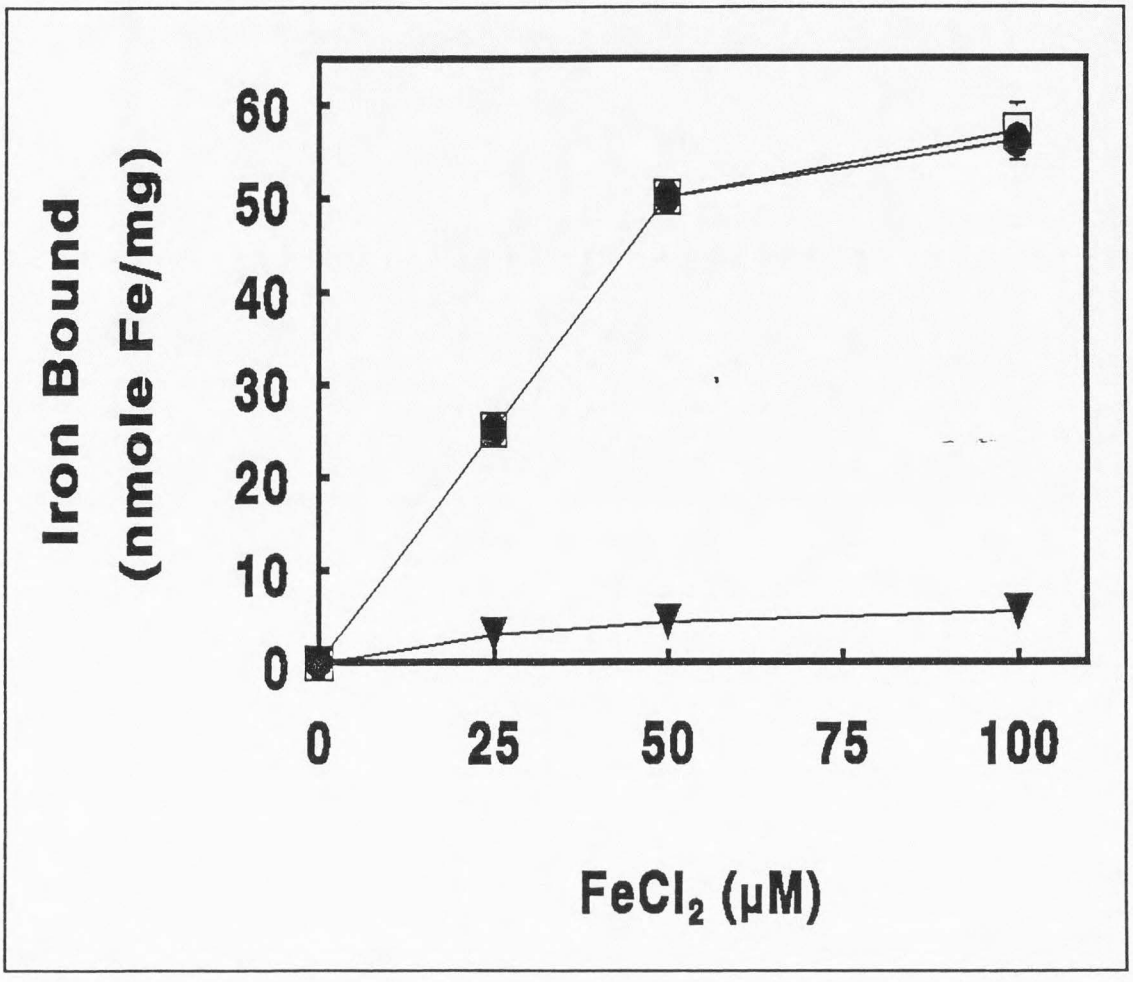

Fig. 6. Ferrous binding by crocidolite from $FeCl_2$ solutions. Native (□) , soaked (●) , or DF crocidolite (▼) were suspended at 1 mg/mL in the indicated concentrations of $FeCl_2$ solutions, the amount of iron bound in 1 hour was determined as described in **Materials and methods**. Data are presented as the mean ± S.D. (n=3). The absence of standard deviation markers indicates that the S.D. is less than the height of the marker.

crocidolite, respectively. Incubation of #1, #2, and #3 in 50μM $FeCl_2$ for 1 h resulted in binding of 6.5±0.4, 29.0±0.5, or 2.7±1.9 nmol Fe^{+2}/mg .

The average surface area of the crocidolite sample used was 10.1 m^2/g (11). By assuming even distribution of bound iron on the surface of the fiber, and knowing the average surface area, the surface coverage of ferrous ions bound to the fibers was calculated. The number of ions bound to the fibers incubated in ferrous chloride solutions ranged from 1.8×10^{17} to 3.5×10^{18} atoms of iron/m^2 crocidolite. DF crocidolite incubated in 25 μM $FeCl_2$ exhibited the lowest surface coverage, and native crocidolite which had been incubated in 100 μM $FeCl_2$ had the greatest surface coverage.

Cations released into the supernatant during the loading process were determined by ICP analysis. Sodium, magnesium, calcium, and trace amounts of manganese, all ions which are known to be constituents of the crocidolite sample used (11), were observed in the supernatant following ferrous loading.

Kinetics of ferrous binding to native crocidolite

To determine the rates at which iron was bound to native crocidolite fibers, binding efficiency was assayed after 5, 15, 30, 60, or 120 min incubation. As depicted by the results in Fig. 7, the most rapid binding phase occurred during the initial 5 min, in all three concentrations of

$FeCl_2$ solutions. The initial binding rate from all iron containing solutions was approximately 5 nmol Fe^{+2}/mg crocidolite/min. The binding rate in all incubation concentrations decreased following the initial 5 min, but iron binding to the crocidolite continued until all of the iron from solution had been bound, except from the 100 μM solution where 3 hours incubation was required for all of the iron to be bound by the fiber (unpublished data). Considering the number of ions bound in the first 5 min to be occupying the sites of highest affinity allowed calculation of the number of binding sites to which ferrous is bound with fast kinetics. These calculations show that 1.49×10^{18} such binding sites exist per square meter of crocidolite.

Effect of additional iron on the ability of crocidolite to catalyze the formation of DNA single-strand breaks

The results in Table 4 demonstrate that in the presence of ascorbate, with or without citrate or EDTA, crocidolite or soaked crocidolite with 0, 25, or 57 nmol Fe^{+2}/mg catalyzed the introduction of the same amounts of DNA SSBs. The binding of additional iron to DF crocidolite, however, did result in significant increases in fiber reactivity. The

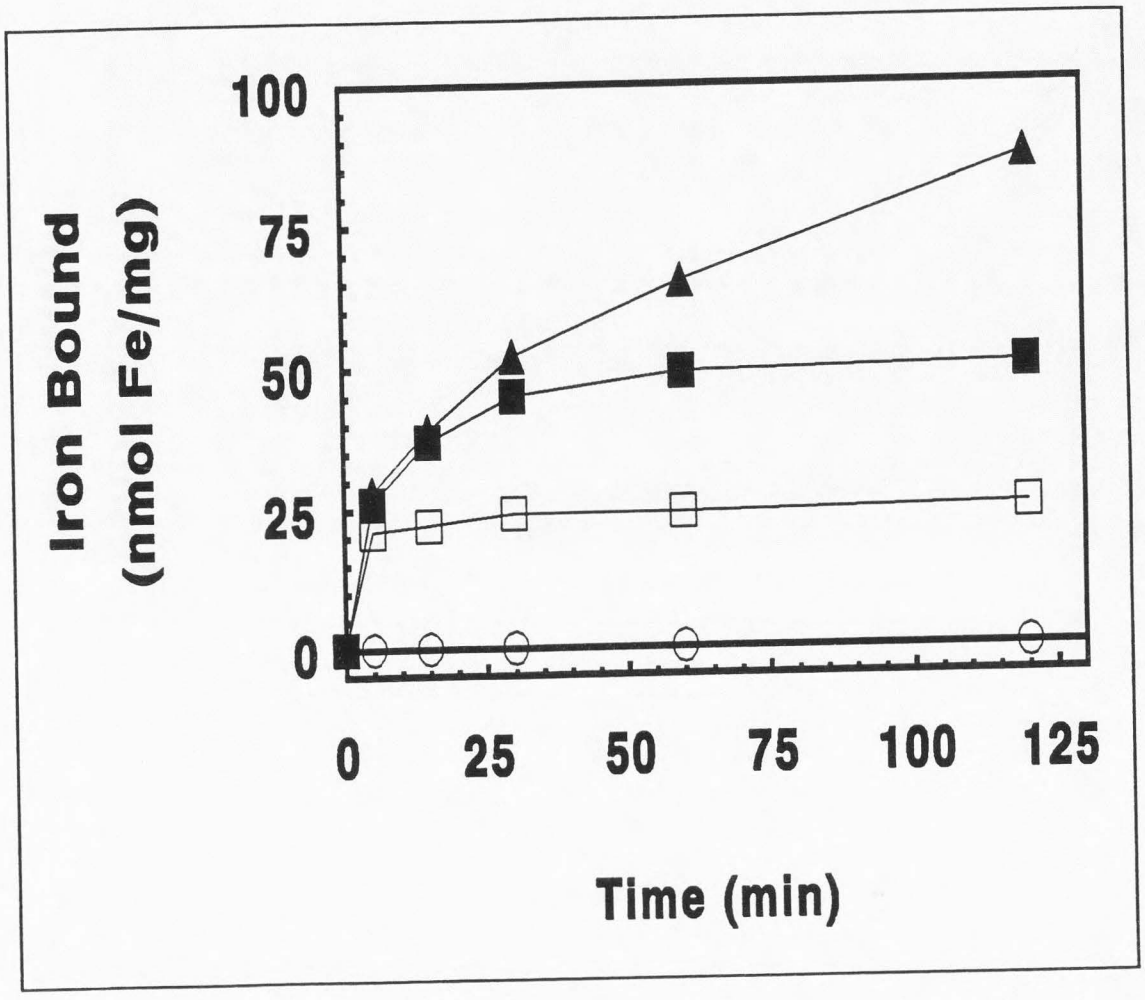

Fig. 7. Kinetics of iron binding to native crocidolite. Native crocidolite was suspended at 1 mg/mL in 0 μM (O), 25μM (□), 50 μM (■) or 100 μM (▲) $FeCl_2$ solutions. The amount of iron bound to the fibers was determined as described in **Materials and methods** at the indicated time points. The experiments were repeated at least two times.

Table 4. Formation of DNA single-strand breaks by native, soaked and DF crocidolite after ferrous binding

	Crocidolite		Soaked Crocidolite		DF crocidolite	
Chelator[a]	Fe^{+2} bound[b]	%DNA SSB[c]	Fe^{+2} bound[b]	%DNA SSB[c]	Fe^{+2} bound[b]	%DNA SSB[c]
	0	17 ± 3	0	11 ± 3	0	8 ± 4
None	25	16 ± 5	25	16 ± 3	3.0	12 ± 3
	57	14 ± 3	56	13 ± 3	5.5	20 ± 3†
	0	38 ± 1	0	43 ± 3	0	12 ± 7
Citrate	25	40 ± 8	25	45 ± 3	3.0	18 ± 7
	57	40 ± 8	56	44 ± 4	5.5	27 ± 7‡
	0	45 ± 5	0	47 ± 3	0	21 ± 3
EDTA[d]	25	52 ± 6	25	48 ± 4	3.0	42 ± 2†
	57	52 ± 4	56	51 ± 4	5.5	58 ± 1†

[a] Incubation conditions included ascorbate (1 mM), chelator (1 mM), 0.25 μg ϕX174 RFI DNA in 50 mM NaCl, pH 7.5.
[b] Iron bound is expressed as nmol Fe^{+2}/mg crocidolite.
[c] Results are expressed as the mean ± SD (n=3) and are relative to the control, untreated DNA.
[d] Native and soaked fibers exposed to EDTA + ascorbate were incubated for 15 minutes. All other incubations were for 30 minutes.
† Significantly different from corresponding fiber before iron binding ($P < 0.01$).
‡ Significantly different from corresponding fiber before iron binding ($P < 0.05$).

total amount of additional iron bound to DF crocidolite was much lower than the amount bound to the native or soaked crocidolite (Table 4). The addition of a chelator, citrate or EDTA, increased the strand break activity of all forms of crocidolite, with EDTA causing the greatest increase. As before, no significant differences were observed between native crocidolite and soaked crocidolite, and additional iron appeared to have no effect. There was, however, a relationship between the amount of iron added to DF crocidolite and the amount of DNA SSBs induced. The addition of 5.5 nmol Fe^{+2}/mg to DF crocidolite more than doubled the amount to SSBs observed when no iron was added. For native, soaked, and DF crocidolite, in the presence of no chelator and no reductant, or chelator only, background levels of single-strand breaks were observed. The single-strand breaking activity before and after iron incubation could be completely inhibited by addition of 1 mM desferrioxamine B to the DNA reaction mixture, indicating that the SSB reactivity was due to iron (unpublished data).

Mobilization of iron from crocidolite has been shown to directly correlate with the amount of SSBs introduced in ϕX174 RFI DNA (21). When this DNA is exposed to crocidolite for longer than 30 min, extensive damage occurs, which disallows quantification of the SSB induced. To observe whether additional iron on the surface of crocidolite may be biologically active at later time points, crocidolite loaded

with 57 nmole Fe/mg was incubated in EDTA or citrate solutions. As shown in Table 5, more iron was mobilized in 4 or 24 hours from fibers which had been treated with iron than from native fibers. Although loaded iron did not confer an enhanced ability to native crocidolite to form DNA single-strand breaks in a short term incubation, additional iron appeared to biologically available through mobilization at longer incubation times.

Effect of additional iron on the ability of SiCWs to catalyze the formation of DNA single-strand breaks

As shown in Fig. 8, both prior to and following incubation in $FeCl_2$ solutions, the three varieties of SiCWs had varying abilities to catalyze the formation of DNA SSBs. In the native condition, SiCW #2 consitently had a greater ability to form DNA SSBs in the presence of ascorbate with or without chelators present. SiCWs #1 and #3 had similar abilities to form DNA SSBs natively (see Fig. 8). Under all incubation conditions, the ability of the fibers to form DNA SSBs was markedly increased following binding of iron. This is particularly surprising in light of the fact that so little iron was actually bound from solution by the SiCWs themselves. In the presence of ascorbate only, #3 had the greatest increase in ability to form DNA SSBs (34%) followed by #1 (29%) and #2 (11%), respectively. When both the chelator citrate and the reductant ascorbate were added to

Table 5. Mobilization of iron from iron-treated crocidolite by citrate and EDTA

	Iron mobilized (nmol Fe/mg crocidolite)[a]			
Time	Citrate		EDTA	
	Native	Iron-Treated	Native	Iron-Treated
4 h	9 ± 0.1	11 ± 0.2‡	31 ± 0.4	42 ± 0.2‡
24 h	24 ± 0.4	38 ± 0.1‡	57 ± 0.6	85 ± 0.1‡

[a] Results are expressed as the mean ± SD (n=3).
‡ Significantly different from fiber before iron treatment ($P < 0.05$ Student's *t* test).

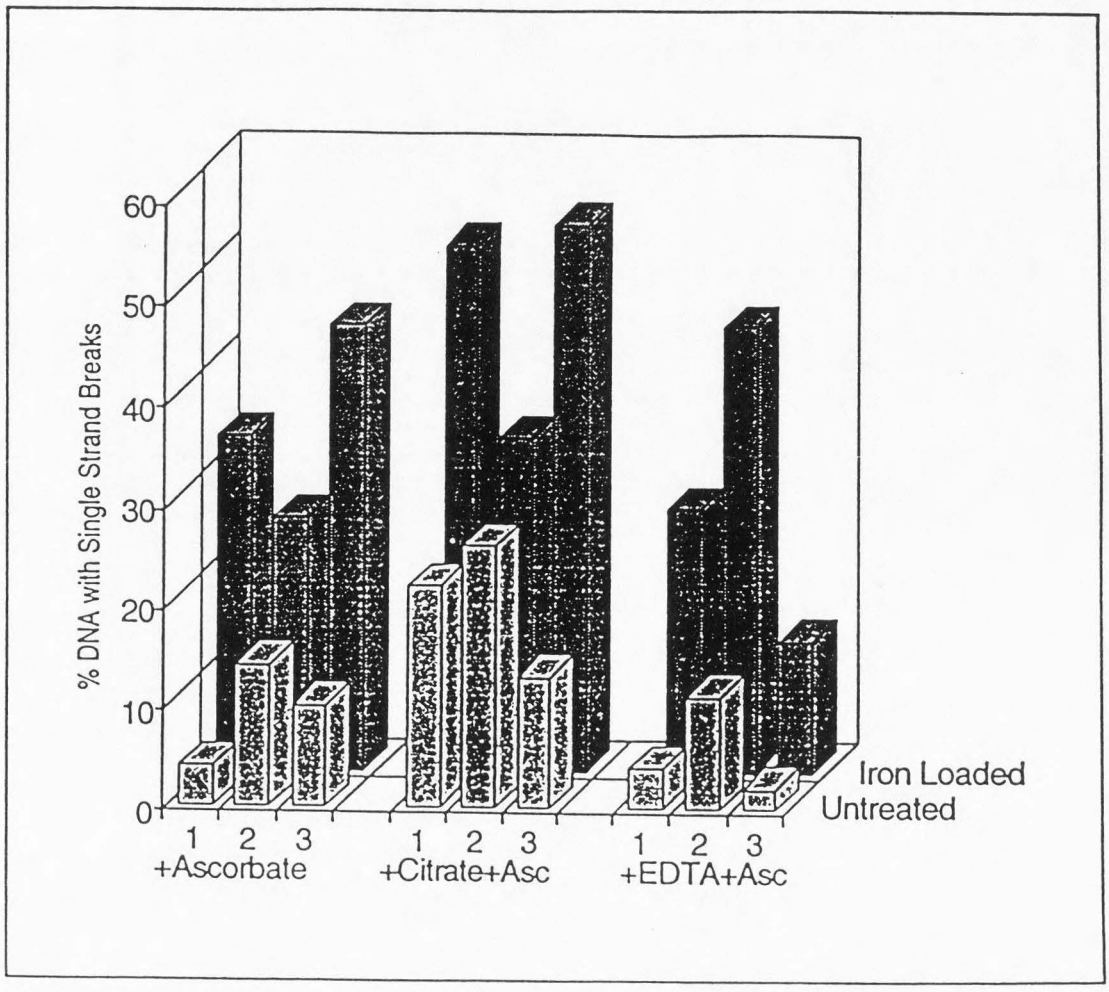

Fig. 8. The effect of iorn binding on the ability of silicon carbide whiskers to catalyze the formation of DNA single-strand breaks. Silicon carbide whiskers #1, #2, and #3 in their untreated (light grey bars) and iron loaded condition (dark grey bars) were exposed to DNA in the presence of ascorbate alone, or with EDTA or citrate as described in Materials and methods. The data are shown as the mean (n=3) percent DNA with single-strand breaks.

the incubation mixture with DNA, SiCWs #1, #2, and #3 exhibited increases to form DNA SSBs by 30, 7, and 41%, respectively. When EDTA and ascorbate were used, the increases in the abilities of the fibers to form DNA SSBs were 22, 33, and 9%, respectively. In the presence of ascorbate only, or citrate and ascorbate, SiCWs #1 and #3 were more able to damage DNA after incubation in $FeCl_2$ than was #2, which natively had the greatest damaging capability. In the presence of EDTA and ascorbate, #2 had the greatest ability of the three varieties to damage DNA both before and after iron was bound.

Effect of incubation in tissue culture medium on DNA SSB formation by crocidolite

In order to assay the ability of crocidolite and DF crocidolite to acquire iron from a complex, more physiologically relevant solution, crocidolite fibers were incubated in F-12 or Fe F-12 tissue culture medium. The reactivity of the fibers incubated in medium was assayed by the DNA SSB assay. Native and DF crocidolite were used in this study because no differences had been observed between soaked and native crocidolite in the previous study. As shown by the results in Fig. 9, both native and DF crocidolite showed enhanced reactivity in the DNA SSB assay after incubation in an iron-containing tissue culture medium compared with incubation in an iron-free medium. The

enhanced reactivity of native crocidolite was only observed in the presence of ascorbate. The increases in DNA SSB activity of native crocidolite after incubation in iron-containing medium were 5, 3, or 7% under assay conditions of ascorbate alone, ascorbate and citrate, or ascorbate and EDTA, respectively. When DF crocidolite was incubated in iron-containing medium, the fibers exhibited an increase in DNA SSB activity of 6, 13, or 24% when assayed under the same conditions. When the assay was performed with a chelator in the absence of a reductant or in the absence of a chelator and a reductant, background levels of DNA SSBs were observed (unpublished data). The ability of native crocidolite or DF crocidolite to induce single-strand breaks under all conditions was completely inhibited by addition of desferrioxamine B to the reaction mixture (unpublished data).

Discussion

Results shown here indicate that native, soaked, or DF crocidolite or SiCWs are capable of acquiring additional iron from simple $FeCl_2$ solutions or from more complex solutions, such as tissue culture medium. The finding that iron can be bound by crocidolite from tissue culture medium suggests that binding of iron directly to the fiber would indeed be feasible from intracellular chelators, which may

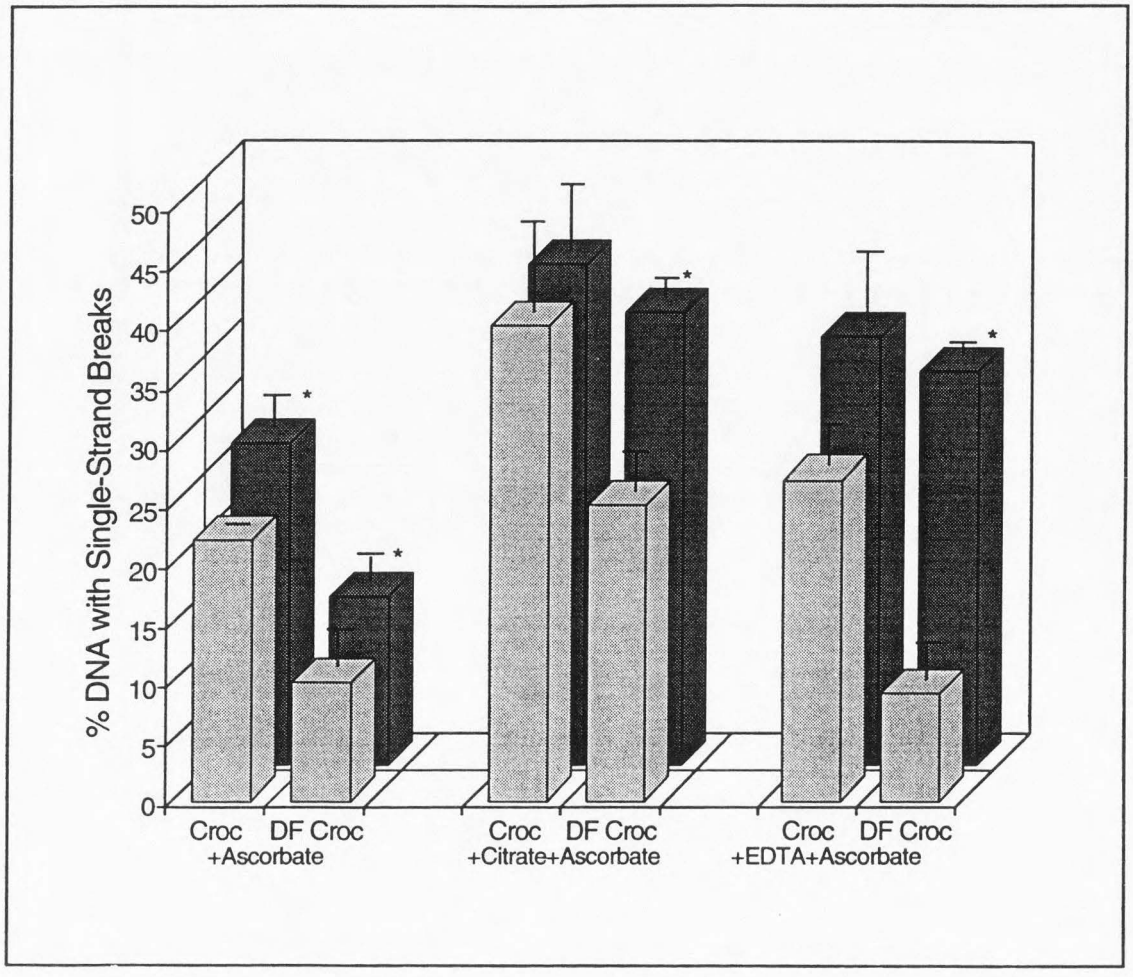

Fig. 9. Effect of incubation in tissue culture media on the ability of native and DF crocidolite to catalyze DNA single-strand breaks. Crocidolite (Croc) and DF crocidolite (DFCroc) were incubated for one hour in F-12 media (light grey bars) or Fe F-12 (black bars). The fibers were subsequently incubated with DNA in the presence of ascorbate with or without citrate or EDTA, as described in **Materials and methods**. The data are shown as the mean ± S.D. (n=3) percent DNA with single-strand breaks.

be very similar to those found in tissue culture medium. Organic acids, intermediates in metabolic processes, and amino acids are all known to coordinate iron (50-52). Iron binding from simple solutions provides a ready means for studying the kinetics of iron binding. The finding that the most rapid phase of binding occurred immediately suggests that there are a certain number of saturable sites which are active in the early and most rapid binding phase. These sites are likely to be unoccupied coordination sites or rough areas in the microtopograpy of the fiber which appear along cleavage or termination planes in the mineral (53). Other sites which still contain cations are more likely to be involved in the slower phase of iron coordination.

No significant difference was observed in the ability of native or soaked crocidolite to catalyze the formation of DNA SSBs after ferrous binding. Ferrous ions were likely to have been coordinated at the same types of binding sites to which the intrinsic ferrous and ferric ions are coordinated. Ion exchange has been reported to occur on crocidolite under some conditions (54) and may have occurred during ferrous binding, as suggested by the observation of intrinsic cations from crocidolite in the supernatant after ferrous binding. Fig. 10 depicts the M1, M2, M3, and M4 binding sites in the crocidolite crystal structure (55,56). M1, M2, and M3 are generally filled with ions 0.5-0.9 Å in radius such as Mg^{+2}, Fe^{+2}, and Fe^{+3}. Additional ferrous ions (ionic

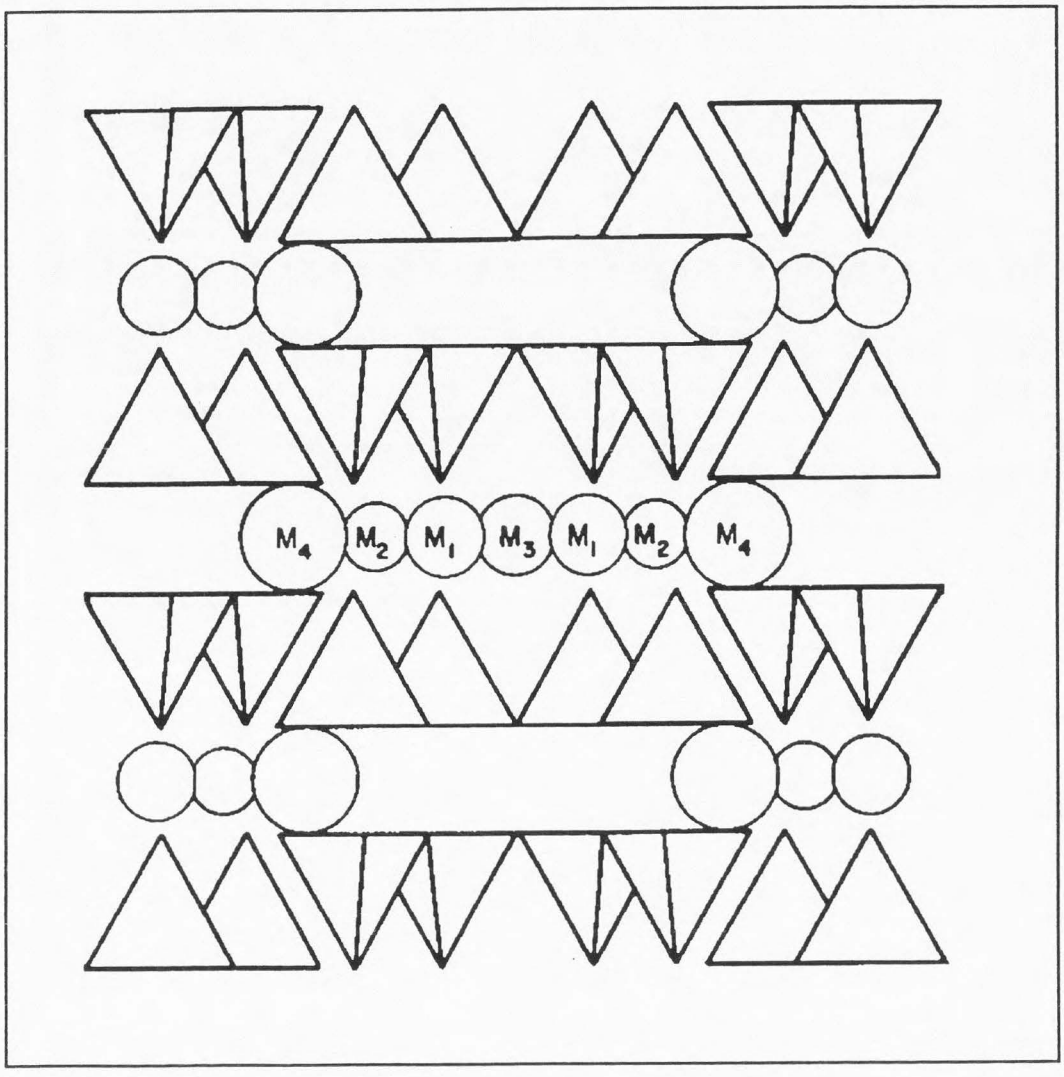

Fig. 10. Structure of crocidolite. This is an idealized diagram of the structure of crocidolite, $[Na_2Fe^{(III)}{}_2Fe^{(II)}{}_3Si_8O_{22}(OH)_2]$, after that of Earnst (44). Triangles represent the silicon-oxygen tetrahedra which are linked together into infinite one-dimensional chains. Triangles containing vertical lines are the tetrahedra coming out of the plane. Empty triangles are tetrahedra directed into the plane. The circles depict the various cation binding sites (M_1, M_2, M_3, and M_4), labeled only in the center of the diagram, which are present between the silicate chains within the crocidolite structure.

radius 0.76 Å) would be likely to replace Mg^{+2} ions or fill M1, M2, or M3 sites that have been vacated along cleavage planes in the mineral fiber. M4 coordination sites are generally occupied by ions 0.7-1.1 Å in radius. Sodium ions are the principal candidates from the idealized structure of crocidolite for the M4 binding site. Ferrous ions are likely to exchange with Na^{+1} cations from these M4 coordination sites. In the presence of a chelator, as in the DNA SSB assay, the additional ferrous ions may have been mobilized from the native or soaked crocidolite at the same rate that intrinsic iron was mobilized from the fiber before treatment because the additional ferrous ions would have become indistinguishable, in terms of coordination, from the intrinsic ferrous ions.

The important effect of iron binding is that the total iron content increased as the fiber was incubated in iron-containing solutions. Mobilization by EDTA from iron-treated fibers was significantly greater than from native fibers in 4 or 24 h. This finding suggests that, indeed, additional iron could have a significant impact on the long-term biochemical reactivity of the fiber, although an increase in the amount of DNA SSBs at very early time points (15 or 30 min) was identical for iron-treated and native crocidolite. If the fiber were continuously exposed to conditions favoring iron binding intracellularly, any available coordination sites could become filled and the fiber could

be enriched with iron. Enriched regions could serve as additional sources of iron to increase the total amount of iron available for long periods of time and could subsequently serve as the nucleation sites for crystal growth in ferruginous body formation (57,58). In studies with erionite, another carcinogenic mineral fiber, nucleation has been proposed as a necessary process in the addition of large amounts of iron to the fiber (41).

DF crocidolite exhibited an increased ability to catalyze the formation of DNA SSBs after ferrous ions were bound. This finding is similar to that of Adachi *et al.*, who reported that DF crocidolite had an increased ability to form 8-hydroxydeoxyguanosine in the presence of an additional iron source (59). The surface cation binding sites would have been vacated due to the chelation treatment to render the fiber suface iron-depleted (DF crocidolite). Desferrioxamine B has been reported to bind crocidolite during chelation treatment (60). Binding of desferrioxamine B is likely to have occurred during the pretreatment described here, and could have complexed iron still associated with the fiber. Desferrioxamine B molecules associated with the fiber in this way are likely to sterically hinder binding of additional iron atoms. This may explain why DF crocidolite bound less iron than native crocidolite. Iron bound to desferrioxamine B is not capable of reducing O_2; therefore, reactions of desferrioxamine B-

bound iron with DNA will not occur. An increase in SSB reactivity was observed after ferrous treatment, which indicates that additional iron bound to the fiber, rather than to desferrioxamine B that may have been associated with the fiber. It is probable that iron bound to coordination sites which were not occupied by desferrioxamine molecules, and therefore was readily available for chelation and mobilization by EDTA or citrate when exposed during the DNA SSB assay. The effects of this additional iron may be particularly observable because the background fiber is largely iron-free. During the slow, but observable dissolution which occurred during chelation treatment, the rapidly filled coordination sites along cleavage planes are likely to have been among the 1% of the fiber that dissolved (53). Based on the amount of iron that was bound to the DF crocidolite in 1 h, it appeared that the slow-phase binding occurred on DF crocidolite, rather than the rapid binding process. A very small quantity of iron was bound to the DF crocidolite, compared to the amount which bound to native crocidolite. This amount of iron significantly increased the ability of the fiber to cause biological damage, indicating once again that small changes in the iron content of the fiber could have notable repercussions on the lifetime biochemical reactivity of inhaled fibers. Intracellularly, crocidolite that has had iron removed may become re-enriched with iron under iron binding conditions, and regain former

or, at least somewhat, elevated damaging potential.

Indeed it appears that fibers may introduce a new factor into the dynamic intracellular system after inhalation. It has been proposed that durable fibers with cation-binding capabilities may upset normal intracellular iron metabolism (18,24). Under certain conditions iron is mobilized from the fiber (23). This event may increase the damaging potential of the iron because it allows direct contact with fragile biomolecules throughout the cell. The singlular difference between F-12 and Fe F-12 is the iron content. The observation that both crocidolite and DF crocidolite were more capable of causing DNA SSBs after incubation in Fe F-12 strongly suggests that the fibers are able to bind iron from a tissue culture medium. This further suggests that, in an aqueous environment like that of the cell, or extracellular space, inhaled durable fibers may also be able to bind reactive iron which could potentiate the dangerous nature of the fiber. This may occur when the fiber is able to carry the newly acquired iron from cells, such as macrophages, to target cells, catalyzing tumor formation. It should likewise be noted that iron, and conceivably other components contained in tissue culture medium, can bind to and affect the reactivity of fibers. Events such as these may have serious effects on experiments performed in culture medium.

These results have demonstrated that iron can be bound

at the same rate to crocidolite fibers in their native, freshly suspended form, and after soaking for 90 days in a nonmobilizing, aqueous environment. It is well known that the dissolution kinetics of the silicate structure amphiboles, such as crocidolite, are very slow (53). These results suggest that the early dissolution, which occurs within the first 3 months after suspension in aqueous condition, has a negligible effect on the reactivity of the intrinsic iron on the fiber. The ability of the fiber to acquire iron from solution and to catalyze the formation of DNA SSBs was not affected by aqueous suspension, suggesting that no gross structural changes occurred to the fibers as a result of aqueous suspension alone.

When incubated in an iron-depleting environment, changes in the fiber occurred which decreased the ability of crocidolite to bind iron from solution as well as to catalyze the formation of DNA SSBs. Additional iron bound to DF crocidolite increased its ability to catalyze the formation of DNA SSBs *in vitro*, suggesting that additional iron on the surface of a mineral fiber may lead to enhanced biochemical reactivity of the fiber toward a number of biological targets. The ability of crocidolite and DF crocidolite to bind iron from solution correlated with the cytotoxic capabilities of the fibers in RL-82 cells (28), peritoneal macrophages and P388D cells (29), and Syrian hamster embryo cells (27). The iron-binding capabilities

also correlated with the ability of the fibers before iron binding to catalyze the formation of DNA single-strand breaks *in vitro* (27). Fibers with a greater ability to bind iron also posess a greater potential to damage DNA or to cause cytotoxicity in cells. This correlation may suggest a scheme by which the potential hazard of both man-made fibers and naturally occurring mineral fibers, like crocidolite, to biological systems may be assessed. The ability of a fiber to acquire iron from solution, which may be mobilized subsequently, as determined by the DNA SSB assay for low intrinsic iron content fibers or by mobilization from fibers with intrinsically high iron content, may be predictive both of the cytotoxicity and DNA SSB ability of fibers.

The abilities of the three varieties of SiCWs to form SSBs after iron binding was particularly astounding in light of the fact that the amount of increase in the abilities of the fibers to induce DNA SSBs was not coordinated with either their ability to bind iron or the amount of iron bound by the fibers in 1 hour. SiCW #2 behaved similarly to crocidolite. After iron binding, the fiber had an increased ability to form DNA SSBs, which was most noted in the presence of EDTA and ascorbate, and less accentuated in the presence of citrate and ascorbate. Although of the three varieties SiCW #3 bound the least amount of iron from solution, it exhibited the greatest increase in ability to form SSBs after incubation in iron-containing solutions.

This may suggest that an entirely different mechanism exists for SiCWs than for crocidolite asbestos. Perhaps another transition metal may be present as an inclusion in the SiCWs. This transition metal could be reduced by incubation in ferrous iron solutions and activate the fibers toward reduction of O_2. This is reasonable since Fe^{+2} is known to be able to reduce metals to an unusual oxidation state under various conditions. Additional transition metals were not present in the SiCWs at levels above background by scanning electron microscopy analysis or by acid leaching and ion analysis by ICP (data not shown). However, if more fibers were available, digestion of a large amount of the fibers may allow quantitation of contaminating metals that may be responsible for the *in vitro* reactivity of these SiCWs. Based on these results alone, SiCW #2 would probably have the greatest biological activity, because of its ability to bind reactive iron from solution.

In summary, analysis of the results presented here suggests that all three states of crocidolite studied, native, soaked, and DF, as well as SiCWs, were capable of acquiring additional ferrous ions from solution. Additional iron, when measured against a low iron background, like that in DF crocidolite, appeared to enhance the reactivity of the fiber towards DNA. Additional iron or simply the interaction of the reducing environment like the $FeCl_2$ increased the ability of all three types of SiCWs to form DNA SSBs.

Additional iron could lengthen the reactive lifetime of the fiber, leading to an increase in the damage induced by that fiber. Ultimately, the ability of any fiber to bind iron in a form that is reactive on the surface or easily mobilized to become reactive may determine its biological activity in laboratory animals and in man.

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CHAPTER 3

INFLUENCE OF IRON IN TISSUE CULTURE MEDIA ON CROCIDOLITE-DEPENDENT CYTOTOXICITY AND DNA SINGLE-STRAND BREAKS[1]

Abstract

The cytotoxicity of crocidolite and crocidolite that had been treated with desferrioxamine-B to remove iron (DF crocidolite) was assayed in A549 cells cultured in different tissue culture media containing various amounts of iron. The ability of the fibers incubated in the various tissue culture media to cause DNA single-strand breaks (SSBs) was also determined. Crocidolite cultured in an iron-free medium exhibited less cytotoxicity than in iron-containing media. DF crocidolite had a differential cytotoxic capability from native crocidolite in iron-free media, but the cytotoxicity of the two fibers in iron-containing media was nearly identical. Incubation of the fibers in these media resulted in changes in the abilities of the fibers to catalyze the formation of DNA single-strand breaks (SSBs). DF crocidolite was quite sensitive to incubation in iron-containing media. Incubation in two varieties of media containing iron resulted in increased ability to form SSBs over fibers incubated in the iron-free medium. Crocidolite was also susceptible to changes in its ability to cause DNA SSBs, and

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[1] Coauthored by Jeanne A. Hardy, Chien-Chung Chao, and Dr. Ann E. Aust

incubation in several iron-containing media resulted in increases in DNA SSB ability. A strong correlation was observed between the cytotoxic capabilities of crocidolite to cells cultured in iron-free medium, medium containing 0.25 μM Fe, or medium containing 3 μM Fe and the ability of the fibers incubated in those media to catalyze the formation of SSBs in DNA *in vitro*. The fiber characteristics and the resultant mechanisms that are responsible for the generation of DNA SSBs *in vitro* may also be operative in the cytotoxic mechanism intracellularly. These findings suggest that choice of tissue culture medium could have profound effects on the results observed in asbestos treatment because fibers such as crocidolite may be capable of binding additional iron from the medium in which they are suspended.

INTRODUCTION

Crocidolite asbestos is known to be amongst the most carcinogenic mineral fibers. It is recognized to cause mesothelioma, the hallmark of asbestos exposure, as well as bronchogenic carcinoma, and carcinoma of the stomach and esophagus (reviewed in 1,2). Crocidolite is in the amphibole family of mineral fibers and contains 27% iron by weight (3). Crocidolite is known to cause many of the same deleterious effects as iron, namely generation of oxygen radicals (4-6), oxidation of DNA bases (7-9), strand breaks in DNA (10), and lipid peroxidation (11) both *in vitro* and *in vivo*. Recently it has been proposed that many of the

results of crocidolite exposure stem from mobilization of intrinsic iron to locations within the cell where it can damage biomolecules (12,13). Iron has been reported to be mobilized intracellularly from crocidolite in A549 cells (14). Crocidolite that has been treated with the iron chelator desferrioxamine-B for 90 days (DF crocidolite), a treatment which removes approximately 7% of the intrinsic iron, has shown a lower toxicity to Syrian hamster embryo cells than native crocidolite (15). Incubation of crocidolite with desferrioxamine-B for various periods of time resulted in less toxicity to RL-82 rat lung fibroblasts (16), mouse peritoneal macrophages, and $P388D_1$ cells (17). Desferrioxamine-B has been effective in completely inhibiting the formation of DNA single-strand breaks (SSBs) induced by crocidolite (12), and DF crocidolite exhibited a diminished ability to induce SSBs in DNA compared to native crocidolite (18). Mobilization of iron from crocidolite has been shown to be important in generation of oxidative damage. Iron mobilization has been shown to correlate with crocidolite-dependent DNA SSBs (12), O_2 consumption (13), and $\cdot OH$ production (19).

In addition to mobilization from fibers, iron is also known to bind to fibers after long-term residence inside the lungs of exposed persons. Fibers that have acquired thick layers of deposited iron are called ferruginous bodies (20-22). The iron on ferruginous bodies appears to be

biochemically reactive, as it was found to be capable of catalyzing the formation of SSBs in DNA (23). It has recently been reported that fibers injected intrapleurally in rats acquired iron on their surfaces within days after exposure and that fibers exposed to $FeCl_3$ solutions acquire iron on their surfaces (24,25). Crocidolite and DF crocidolite have also been reported to acquire biochemically reactive iron from $FeCl_2$ solutions (18). Another carcinogenic mineral fiber, erionite, also appears to be capable of binding biochemically reactive iron from both $FeCl_2$ and $FeCl_3$ solutions (26). Acquisition of iron from sources inside the body or from *in vitro* sources appears to affect the reactivity of fibers (18, 24-26).

Much of the research being performed on carcinogenic mineral fibers involves tissue culture experiments. Cytotoxicity has been used to measure the biological effect of many compounds; however, the target molecule for cytotoxicity is not known. If fibers can acquire biochemically reactive iron, the results of the experiments could be strikingly influenced by the selection of the medium in which cells are grown. Experiments to determine the cytotoxicity of asbestos fibers have given variable results, depending on the cell line and type of tissue culture medium employed (27).

The experiments described here were undertaken to determine the effect of iron in tissue culture media on the

ability of the fibers to induce DNA SSBs *in vitro* and the cytotoxicity of the fibers in cultured cells. Human lung carcinoma cells (A549) were cultured in various tissue culture media, and treated with crocidolite and DF crocidolite in cytotoxicity assays. The fibers were also incubated in tissue culture media and exposed to DNA in order to measure the abilities of the fibers to form DNA SSBs. The presence of iron in tissue culture media was related to the cytotoxicity observed. A correlation between the cytotoxicity of crocidolite in A549 cells and the ability of crocidolite incubated in the corresponding media to catalyze the formation of DNA SSBs *in vitro* was also observed. The addition of iron to iron-free medium also enhanced the abilities of fibers to catalyze the formation of DNA SSBs following media incubation.

MATERIALS AND METHODS

Asbestos and reagents. Crocidolite asbestos was obtained from Dr. Richard Griesemer, NIEHS/NTP (Research Triangle Park, NC) and contained 27% iron by weight (3). Closed-circular, superhelical ϕX174 RFI DNA was obtained from New England Biolabs (Beverly, MA). Ethidium bromide, crystal violet, and the sodium salt of L-ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Chelex 100 was obtained from Bio-Rad Laboratories (Richmond, CA). Ferrous sulfate hexahydrate, sodium chloride, the disodium salt of EDTA, sodium bicarbonate, potassium chloride, sodium

phosphate, potassium phosphate, D-glucose anhydrous, and sodium citrate dihydrate were obtained from Mallinckrodt Inc. (Paris, KY). Glass-distilled dimethyl sulfoxide (DMSO) was obtained from Burdick and Jackson (Muskgon, MI). Agarose was obtained from FMC BioProducts (Rockland, ME). Desferoxamine mesylate USP (desferrioxamine-B, N-[5-[3-[5-(aminopentyl)hydroxy-acetamide)pentyl]carbamoyl] propionohydroxamic acid monomethaesulfonate) was obtained from CIBA (Summit, NJ). All remaining reagents were purchased in the highest purity possible.

All NaCl solutions were Chelex-treated to remove contaminating iron before use in the following experiments. DNA was removed from the shipping buffer by ethanol precipitation, as described previously (18), before dissolution in 50 mM NaCl, pH 7.5. Ascorbate solutions were prepared immediately prior to use. All *in vitro* experiments were performed in darkness, under red light to prevent the photochemical reduction of iron (28).

Preparation of Crocidolite. Crocidolite was weighed and suspended in solution immediately prior to use. DF crocidolite was treated with desferrioxamine-B (1mM) for 3 months, as previously described, to remove approximately 7% of the total iron content from the fibers (29). DF crocidolite was stored under sterile conditions in ddH_20 until use.

Cell Culture. A specially prepared lot of Hams F-12

tissue culture medium, free of added iron salts (F-12), Ham's F-12 medium (FeF-12), Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), and sodium pyruvate, and L-glutamine were obtained from Gibco BRL (Grand Island, NY). Fetal bovine serum and Iscove's Modified Dulbecco's Medium (IMDM) were obtained from Hyclone Laboratories (Logan, UT). Gentamicin was obtained from Whittaker, M.A. Bioproducts Inc. (Walkersville, MD). The proper amount of $FeSO_4 \cdot 6H_2O$ was added to powdered MEM or IMDM before dissolution to generate FeMEM and FeIMDM, which both contain 3 μM iron. For the duration of the chapter, the term iron-free refers to media which are free of additional $FeSO_4 \cdot 6H_2O$. The iron-free media used in cell culture experiments were complete growth media and did contain iron bound to transferrin in the fetal bovine serum.

Complete growth medium was composed of the media listed previously, 50 μg/ml gentamicin, 10% fetal bovine serum (Hyclone, Logan, UT), and an amount of sodium bicarbonate that resulted in a final pH of 7.4. The amount of sodium bicarbonate (g/L medium) was 3.024 for IMDM, 3.7 for DMEM, 2.2 for MEM, and 1.176 for F-12. Glucose (1.0 g/L), sodium pyruvate (0.110 g/L), and l-glutamine (0.584 g/L) were also added to DMEM. The atmosphere was 5% CO_2 with 95% humidity.

The complete growth medium with 10% glass-distilled DMSO was used to suspend cells for storage in liquid nitrogen. The cell line A549 (ATCC # CCL185), which is a

human bronchoalveolar carcinoma-derived line, the cells of which have synthetic and morphologic characteristics of alveolar epithelial type II cells (30), was obtained from American Type Culture Collection (Rockville, MD). Upon arrival, cells were thawed, resuspended in complete growth medium, and grown in a Forma Scientific model 315B water-jacketed incubator (Marietta, OH) at 37 ± 1°C in an atmosphere of 5 ± 0.5% CO_2 and 95% humidity. Before the cells reached confluence, they were dislodged with 0.5% trypsin in 0.2% EDTA (Gibco BRL, Grand Island, NY), resuspended in complete growth medium, and plated. For storage, the cells were dislodged, while in logarithmic growth, resuspended in freezing medium as 1 ml aliquots at $2x10^6$ cells/ml, and frozen in liquid nitrogen. Aliquots of A549 cells were removed from liquid nitrogen, thawed, and subcultured in complete growth medium for the experiments.

Preparation of Crocidolite for Treatment of Cells. DF crocidolite was removed from ddH_2O and suspended at 1 mg/mL in sterile $NaHCO_3$. Crocidolite was also suspended at 1 mg/mL in sterile $NaHCO_3$ immediately before use, vortexed for 1 min, and diluted to the appropriate concentration with complete growth medium. Neutron-activated crocidolite was prepared as previously described (6).

Cytotoxicity Assay. The cells were cultured in flasks until 90-95% confluent, then dislodged with 0.5% (V/V) trypsin-0.2% EDTA (W/V) (Gibco BRL, Grand Island, NY),

resuspended in the appropriate complete growth medium, counted using a hemacytometer, and plated in six 60-mm culture dishes at cloning density (200 cells/dish) for each treatment condition. After 6 h, the medium was removed and cells were treated with the indicated concentrations of crocidolite or DF crocidolite. Cells were incubated without disturbance for 10 days, then stained with 0.1% (V/V) crystal violet in methanol. The percent survival of the crocidolite-treated cells was determined by dividing the average number of surviving colonies in the cells treated with crocidolite by the average number of colonies in the control, untreated cells, and multiplying by 100. The average cloning efficiency (colonies observed per cell plated, multiplied by 100) of untreated cells was 50%. Experiments were repeated at least once and reported as the average $\pm$ standard deviation (n=3) or average of two experiments (less than 5% difference between experiments).

Phagocytosis of Crocidolite by A549 Cells. Cells were treated with crocidolite radiolabeled with ^{55}Fe through neutron-activation, dislodged, resuspended in PBS, and counted using a hemacytometer. The cell suspension was centrifuged at 200 x g at 4°C for 10 min, using a refrigerated Beckman GPR clinical centrifuge. The amount of radioactivity associated with the cell pellet was measured using a Beckman LS 5801 scintillation counter. The radiospecific activity of the crocidolite was used to

correct the DPM to μg of crocidolite, and the results are expressed as μg of crocidolite/10^6 cells.

Induction of DNA Single-Strand Breaks by Crocidolite. Crocidolite and DF crocidolite were suspended in various tissue culture media at 10 μg/mL, which is the same concentration of crocidolite in tissue culture medium as when cells are treated. The suspensions were incubated in the dark for 1 h on a horizontal shaker, centrifuged at 160,000 x g in a Beckman Ultracentrifuge Sw40Ti swinging bucket rotor to separate fibers. Because of the low concentration of fibers in the media, it was necessary to use a high centrifugal force to obtain a tight enough pellet to enable removal of the tissue culture media without disturbing the pellet. After removal of the culture media, fibers were suspended at 1 mg/mL. The fibers were then washed five times in NaCl (50 mM, pH 7.5) by centrifugation at 11,000 x g for 3 min and resuspended in NaCl (50 mM, pH 7.5). Twenty μg washed fibers were incubated with 0.25 μg ϕX174 RFI DNA. The SSB assays reported here were performed in the presence of 1 mM ascorbate, with or without 1 mM citrate or 1 mM EDTA as reported previously (12). Incubations of crocidolite with EDTA were for 15 min in the dark, all other incubations were for 30 min in the dark. After the incubation, DNA with SSBs was separated from superhelical DNA (RFI), using agarose gel electrophoresis. The amount of DNA with single-strand breaks was quantified

using integrated scanning densitometry as previously described (12).

RESULTS

Phagocytosis of Crocidolite by A549 Cells

The amount of radioactivity associated with cells may be used to assess the rate of phagocytosis after treatment with radiolabled crocidolite and DF crocidolite. Cells were treated with radiolabeled crocidolite or DF crocidolite. The amount of radioactivity associated with the cells was monitored at 3, 9, 12, 15, 18, or 24 h after treatment. Crocidolite and DF crocidolite were phagocytized at essentially the same rate when the treatments were performed in the same tissue culture medium. Phagocytosis of both types of crocidolite in F-12 was slightly more rapid than in IMDM. At the 24-h time point, cells cultured in F-12 had phagocytized 17.4 or 18.1 μg crocidolite or DF crocidolite/10^6 cells, respectively. Cells cultured in IMDM phagocytized 14.0 or 15.1 μg crocidolite or DF crocidolite/10^6 cells. Eighty-three percent of the amount of DF crocidolite phagocytized by cells in F-12 was phagocytized by cells grown in IMDM, and 80% of the amount of crocidolite taken up by F-12-grown cells was phagocytized by IMDM-grown cells.

Cytotoxicity of Crocidolite in A549 Cells In Several Tissue Culture Media

Cells grown in FeF-12 (3 μM Fe), DMEM (0.25 μM Fe), and IMDM (iron-free) were treated with crocidolite or DF crocidolite. The relative survival of A549 cells under different treatment conditions is shown in Fig. 11. Cells grown in DMEM were not treated with DF crocidolite. Cells grown in iron-containing media exhibited higher crocidolite-dependent cytotoxicity than in iron-free media. The relative survival of cells treated with 2 $\mu g/cm^2$ crocidolite decreased as the amount of iron in the tissue culture media increased. The relative survival was 27% in IMDM, 20% in DMEM, and 9% in FeF-12. DF crocidolite exhibited less cytotoxic effect than crocidolite in A549 cells cultured in IMDM. DF crocidolite was 39% less cytotoxic in IMDM than crocidolite itself at 2 $\mu g/cm^2$ treatment. In FeF-12, DF crocidolite exhibited no significant difference in the cytotoxicity from the untreated fibers.

Effect of Incubation in Various Tissue Culture Media on the Ability of DF Crocidolite to Catalyze the Formation of DNA SSB

In addition to having a lower cytotoxic effect than native crocidolite under certain conditions, DF crocidolite has been more sensitive to additional iron because the surface is relatively free of iron compared to native crocidoite. Therefore, DF crocidolite was employed to

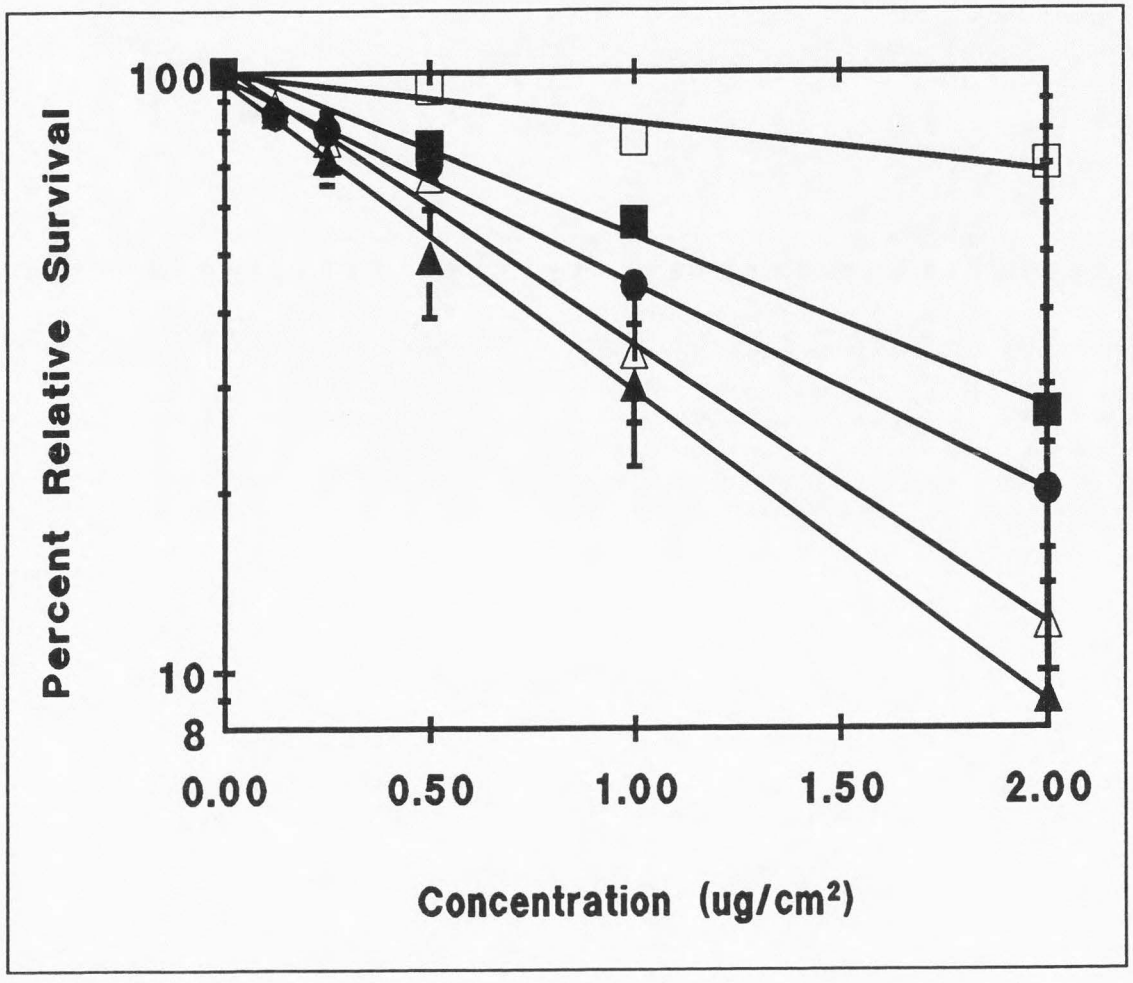

Fig. 11. Cytotoxicity of crocidolite and DF crocidolite in A549 cells cultured in IMDM, DMEM, and F-12. A549 cells were grown IMDM and treated with crocidolite (■) or DF crocidolite (□) or grown in DMEM and treated with crocidolite (●), or grown in F-12 medium and treated with crocidolite (▲) or DF crocidolite (△).

determine the influence of iron in tissue culture media on DNA SSBs. Iron has been shown to bind to crocidolite under various conditions (18,24-26), including exposure to tissue culture media (18). Studies in this field often involve asbestos treatment of cells grown in culture. To determine the effect of incubation in different tissue culture media on biochemical reactivity, DF crocidolite was incubated in water, IMDM, IMDM with 3 μM $FeSO_4$ added (FeIMDM), DMEM, MEM, MEM with 3 μM $FeSO_4$ added (FeMEM), F-12 without iron (F-12), or F-12 containing 3 μM $FeSO_4$ (FeF-12). The DF crocidolite was washed in ddH_2O (four washes) and NaCl (50 mM, final wash) following the media treatment to remove loosely associated components of the media from the fiber. DNA was exposed to DF crocidolite that had been treated with these media. As outlined in Table 6, modifications in the ability of the fibers to catalyze the formation of DNA SSBs were observed after incubation in different media. DNA SSBs formed in the presence of ascorbate only were not significantly different as a result of incubation in various media. In the presence of citrate and ascorbate, incubation in DMEM or FeF-12 increased the ability of the fibers to produce DNA SSBs by 9% or 18%, respectively, over DF crocidolite incubated in water. The ability of DF crocidolite to catalyze the formation of DNA SSBs also increased after incubation in FeMEM or FeF-12 compared to MEM or F-12 by 9 or 8%, respectively. Other components of

Table 6 *Effect of incubation in tissue culture media on the ability of DF crocidolite to catalyze the formation of DNA single-strand breaks*

	% DNA Single-Strand Breaks[a]							
Chelator	Water	IMDM	FeIMDM	DMEM	MEM	FeMEM	F-12[b]	FeF-12
None	7±3	9±5	11±5	7±1	5±4	6±2	8±3	4±5
Citrate	7±5	6±2	ND	16±2[§]	10±3	19±6[†]	25±4[§]	33±2[§‡]
EDTA	23±6	14±5	19±7	9±1[§]	7±2[§]	25±6[‡]	9±3[§]	22±6[‡]

[a] The percentage DNA with SSB is expressed as the mean ± SD (n=3) and is relative to the control, untreated DNA. Assay conditions were in the presence of 1 mM ascorbate with or without 1 mM chelator.
[b] Data taken Hardy and Aust (ref 24).
ND=Not Determined
[§] Significantly different from the corresponding fiber incubated in water ($P<0.05$ Student's *t* test).
[†] Significantly different from the corresponding fiber incubated in the same type of media free of iron. ($P<0.10$ Student's *t* test).
[‡] Significantly different from the corresponding fiber incubated in the same type of media free of iron. ($P<0.05$ Student's *t* test).

the media also seemed to influence the ability of DF crocidolite to cause the formation of DNA SSBs since fibers incubated in two iron-free media, IMDM or F-12, caused SSBs in 6% or 25% of the DNA, respectively.

A similar trend in the pattern of DNA SSB was observed when assays were performed in the presence of ascorbate and EDTA. Incubation in FeIMDM, FeMEM, or FeF-12 increased the ability of the fibers to produce DNA SSB by 5, 18, or 11%, respectively, over fibers incubated in the corresponding iron-free media. When the DNA was exposed to the fibers in the presence of EDTA and ascorbate, there was a decrease in the abilities of the fibers incubated in iron-free media to form DNA SSBs, compared to water incubation. DF crocidolite incubated in the iron-free (IMDM, MEM, F-12) or low iron content media (DMEM) showed similar decreases of 9, 16, 14, or 14% in reactivity towards DNA compared to water-incubated fibers.

Effect of Incubation in Various Tissue Culture Media on the Ability of Crocidolite to Catalyze the Formation of DNA SSB

To determine the effect of tissue culture media incubation on the reactivity of crocidolite, fibers were incubated in previously described tissue culture media, washed, and the reactivity of the fibers was assayed by monitoring the formation of DNA SSBs. The amounts of SSBs introduced by crocidolite incubated in different media are

listed in Table 7. When crocidolite, which had been incubated in tissue culture media, was assayed for the ability to form DNA SSBs in the presence of ascorbate, compared to water-incubated fibers, the fibers incubated in F-12 and FeF-12 had an increased ability to form DNA SSB. When assayed with citrate and ascorbate, decreases of 17, 19, or 5% in SSB activity were observed after incubation in IMDM, FeIMDM, or DMEM compared to water-incubated fibers. Incubation of crocidolite in FeMEM or FeF-12 rendered the fibers moderately more active in the SSB assay than the fibers incubated in the corresponding iron-free media by 8 or 5%, respectively. The fibers incubated in every variety of medium exhibited less SSB activity in the presence of EDTA and ascorbate than the fibers incubated in water. Crocidolite incubated in both FeIMDM or FeMEM showed increases in its ability to cause SSB over IMDM or MEM by 15%, in the presence of EDTA and ascorbate.

Correlation Between Cytotoxicity and DNA Single-Strand Breaks.

Various types of damage to DNA caused by asbestos have been accessed in different systems. The role of DNA SSBs in cytotoxicity and carcinogenicity is not fully understood. Fig. 12 depicts the correlation between crocidolite-dependent cytotoxicity in A549 cells at the highest dose (2 $\mu g/cm^2$) and crocidolite-dependent DNA SSBs *in vitro* in the presence of citrate and ascorbate after incubation in

Table 7 *Effect of incubation in tissue culture media on the ability of crocidolite to catalyze the formation of DNA single-strand breaks*

	% DNA Single-Strand Breaks[a]							
Chelator	Water	IMDM	FeIMDM	DMEM	MEM	FeMEM	F-12[b]	FeF-12
None	14±2	9±6	9±3	15±5	15±4	14±2	22±0[§]	24±2[§]
Citrate	35±2	18±2[§]	16±2[§]	30±1[§]	28±4	36±1[‡]	39±2	44±3[§†]
EDTA[c]	52±2	22±5[§]	37±12	28±4[§]	28±5[§]	43±2[§‡]	29±4[§]	26±3[§]

[a] The percentage DNA with SSB is expressed as the mean ± SD (n=3) and are relative to the control, untreated DNA. Assay conditions were in the presence of 1 mM ascorbate with or without 1 mM chelator.
[b] Data taken from Hardy and Aust (ref 24).
[c] Fifteen minute incubation with DNA. All other incubations with DNA were for 30 minutes in the dark.
[§] Significantly different from the corresponding fiber incubated in water ($P<0.05$ Student's *t* test).
[†] Significantly different from the corresponding fiber incubated in the same type of media free of iron. ($P<0.10$ Student's *t* test).
[‡] Significantly different from the corresponding fiber incubated in the same type of media free of iron. ($P<0.05$ Student's *t* test).

IMDM(iron-free), DMEM (0.25 μM Fe), and FeF-12 (3 μM Fe). The amount of fibers suspended in the various types of media was the same as that administered to cells and the media incubations for the DNA SSB assay. There was a very linear correlation (r^2= 0.996) between the amount of DNA with SSBs and the cytotoxicity to A549 cells.

DISCUSSION

An assortment of experiments has been performed assaying the cytotoxic abilities of various mineral fibers, both man-made and naturally occurring. The results of these experiments have often conflicted with one another because of the variations in cell lines and media (27). The results of the experiments described here indicate that one important factor in the outcome of any cell culture assay of mineral fiber toxicity may be the presence of iron in the tissue culture medium employed because iron from various sources is known to bind to fibers.

Results presented here indicate that there is a strong correlation between the cytotoxicity of crocidolite to A549 cells grown in IMDM, DMEM, or FeF-12 and the DNA SSB ability *in vitro* of crocidolite in the presence of citrate and ascorbate after incubation in the same media. This finding suggests that a mechanism similar to that which is operable in the formation of DNA SSBs occurs intracellularly to cause cytotoxicity in cultured cells. The proposed mechanism for

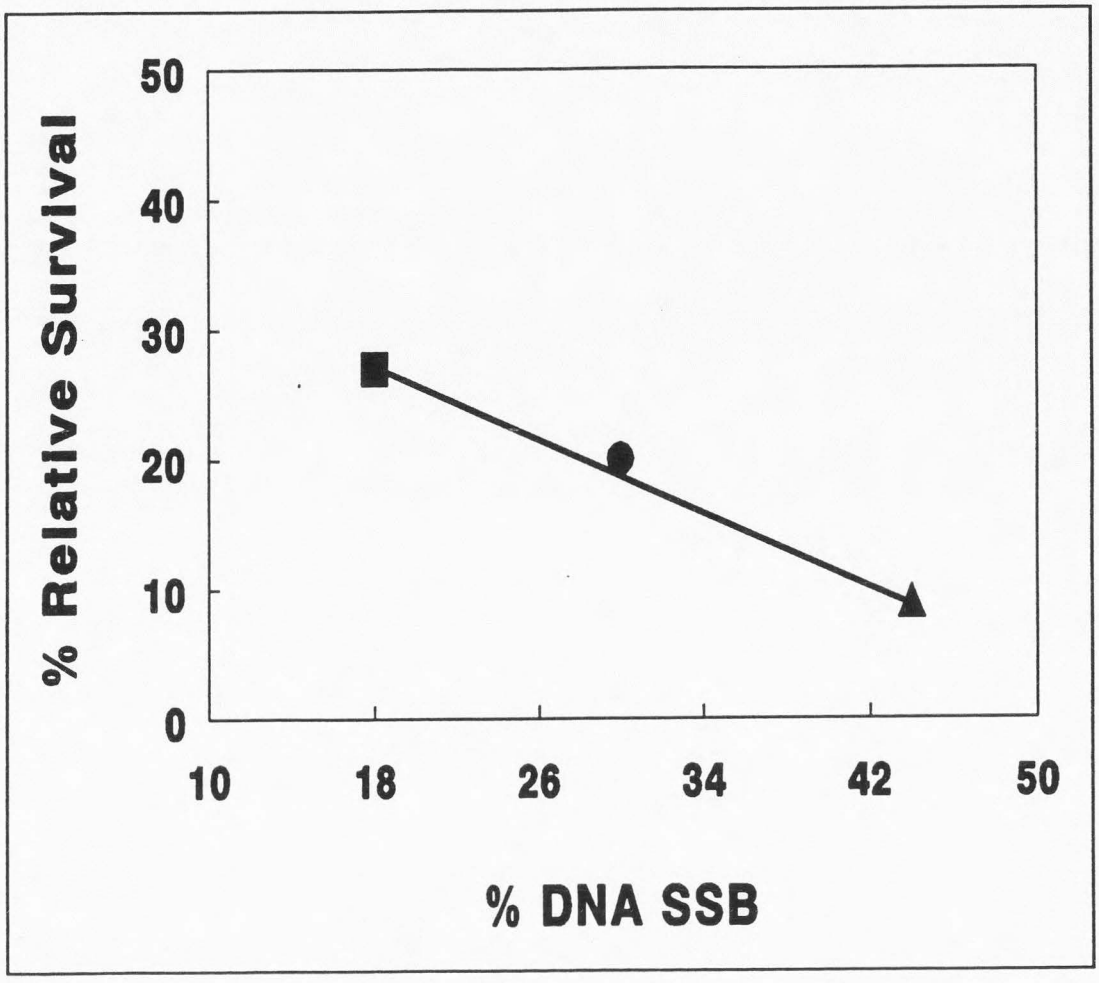

Fig. 12. Correlation of DNA SSBs with cytotoxicity in A549 cells. Cytotoxicity of A549 cells treated with 2 $\mu g/cm^2$ crocidolite in F-12 (▲), DMEM (●), or IMDM (■) was plotted as a function of the ability of fibers incubated in those tissue culture media to catalyze the formation of DNA SSBs in the presence of citrate and ascorbate.

the formation of DNA SSBs *in vitro*, outlined below, involves reduction of molecular oxygen to ultimately form the hydroxyl radical (31).

$$\text{Reductant}^{(n)} + \text{Fe(III)} \rightarrow \text{Reductant}^{(n+1)} + \text{Fe(II)} \quad (1)$$

$$\text{Fe(II)} + O_2 \rightarrow \text{Fe(III)} + O_2^{\cdot -} \quad (2)$$

$$HO_2^{\cdot} + O_2^{\cdot -} + H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

$$\text{Fe(II)} + H_2O_2 \rightarrow \text{Fe(III)} + OH^- + OH^{\cdot} \text{ (Fenton Reaction)} \quad (4)$$

Hydroxyl radical has the ability both to abstract protons from the deoxyribose moieties of DNA and to hydroxylate DNA bases (32). Libbus *et al.* have observed an increase in the number of DNA SSBs upon administration of crocidolite to cultured rat embryo cells (10), and Turver and Brown have reported an increase in DNA strand breaks as detected by S1 nuclease sensitivity after crocidolite treatment in C3H10T½ murine embryonic fibroblastic cells (33). The formation of DNA strand breaks was inhibited by the addition of desferrioxamine-B (33), suggesting that iron was the causative agent in strand break formation. The formation of hydroxyl radical adducts has been shown to have a strong correlation with carcinogenicity (34,35); however, the role of formation of DNA strand breaks in carcinogenicity is less understood. Takeuchi and Morimoto observed a dose-dependent formation of 8-hydroxy-2′-deoxyguanosine upon treatment of human promyelocytic leukemia cell line with crocidolite, which did not correlate with cytotoxicity (36). Our report suggests that the formation of DNA SSBs in cultured cells

may be an important factor in cytotoxicity, whereas it appears that the formation of 8-hydroxy-2'-deoxyguanosine base adducts is not (36).

Cells cultured in IMDM, an iron-free medium, exhibited differential cytotoxic response in the presence of crocidolite or DF crocidolite in human lung carcinoma cells A549. The DF crocidolite was 39% less cytotoxic than native crocidolite. This finding supports previous reports where desferrioxamine-B decreased cytotoxicity in Syrian hamster embryo cells (15), rat lung fibroblasts (16), or murine peritoneal macrophages and macrophage-like cells (17). In the iron-containing medium, FeF-12, cytotoxicity in the presence of native or DF crocidolite was not significantly different. This suggests that iron may have been bound to DF crocidolite, increasing the iron content of the DF crocidolite, and rendering the fibers nearly as reactive as the native crocidolite. As the amount of iron in the media increased, the cytotoxicity of both crocidolite and DF crocidolite increased. This suggests again that the iron in the media had a profound influence on the ability of the fibers to cause damage to the cells.

Some inhibition of the DNA SSB reactivity of crocidolite was observed after incubation in all tissue culture media in the presence of EDTA and ascorbate, but similar inhibition was not observed with citrate and ascorbate. Citrate, of course, is a potential physiological

chelator, but before incubation of crocidolite in tissue culture media, EDTA is a more rapid chelator of iron (31). It appears that mobilization of both acquired and intrinsic iron was important because ascorbate, which is known to mobilize very little iron, did not reveal the same difference in DNA SSBs as when a chelator was employed. Another reason for the differences observed in DNA SSBs or cytotoxicity may be that components of the media in addition to iron may associate with the fiber during the incubation. Desferrioxamine-B has been reported to bind to crocidolite in solution (37). Conceivably, any chelator, or negatively charged molecule, such as phosphate, a component of all the tissue culture media used, could become associated with cations present in the structure of crocidolite. Molecules associated in such a manner may block routes of iron chelation, and by lowering the mobilization from the fiber, concomitantly decrease the generation of oxygen radicals (12,13). Iron associated with the fiber may be more available to chelation by citrate than by EDTA after certain components of the media have associated with the fiber, since the fibers were inactivated in the presence of EDTA, but generally not in the presence of citrate.

A naive view of the interaction of crocidolite with components of aqueous solution would suggest that iron specifically binds to the surface of fibers in suspension. Indeed, it is likely that many compounds may bind or be

mobilized from the fibers under various conditions. Crocidolite in suspension represents a dynamic system which appears to be acutely sensitive to slight changes in its environment, as demonstrated by the fact that incubation in slightly different tissue culture media had profound influence on the oxidative capabilities of the fibers. The one component which was monitored, iron, did appear to be responsible, in many cases, for increasing the reactivity of crocidolite and DF crocidolite toward DNA.

Phagocytosis by cells not traditionally considered to be phagocytic has been previously reported in human muscle fibers (38) and Syrian hamster embryo cells (39). Our results corroborate these reports. The finding that phagocytosis of crocidolite and DF crocidolite occurred at the same rate within the same medium strongly suggests that the results observed were not due to variation in the number of fibers present intracellularly. If corrections are made so that cytotoxicity is compared when the same number of fibers have been phagocytized, there is still a significant difference between the cytotoxicity of 2 $\mu g/cm^2$ treatment in iron-free (IMDM: DF crocidolite 72%, crocidolite 25% relative survival) and iron-containing media (FeF-12: DF crocidolite 18%, crocidolite 13% relative survival).

In summary, a strong correlation was observed between the cytotoxicity of crocidolite to human lung carcinoma cells and the ability of crocidolite to introduce DNA SSBs

after incubation in the same media. The results reported here have demonstrated that the cytotoxicity of crocidolite in A549 cells varied, depending on the type of tissue culture media used. The cytotoxicity was greatest in iron-containing media and least in iron-free media. Further, DF crocidolite and native crocidolite had the same cytotoxic capabilities in iron-containing media, but very different toxicities in iron-free media, suggesting that iron from the medium may have bound to the fiber and changed the reactivity of the fiber. Fibers incubated in various tissue culture media had altered capacities to form DNA SSBs, suggesting that components of the media remained associated with the fibers even after extensive washing. The addition of iron to iron-free media increased the ability of fibers incubated therein to catalyze the formation of DNA SSBs in several instances, suggesting that iron indeed bound to the fiber. These results have demonstrated that care should be taken in the performance of experiments utilizing cell culture to assay the effects of mineral fibers, since the components of the media, such as iron, can significantly affect the reactivity of the fibers.

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CHAPTER 4

SUMMARY

It has been proposed, based on work done in our laboratory, that the reactive iron contained in and on crocidolite fibers is largely responsible for the dramatic carcinogenic effects observed upon exposure to the mineral. Iron mobilization from asbestos has been implicated in many of the reactions catalyzed by crocidolite, namely oxygen consumption, generation of hydroxyl radicals, and introduction of DNA SSBs. If reactive iron is responsible for the carcinogenicity of asbestos, the repercussions of iron acquisition on a fiber may be several fold: i) The reactive lifetime of the fiber is increased with each additional atom of iron which can subsequently be mobilized from the fiber. ii) Entrance of the fiber may represent an even greater abnormal entry of iron into the cell than native fibers. iii) The acute mechanism by which fibers form ferruginous bodies may be modeled.

Inhaled fibers appear to be a dynamic system. Under certain conditions iron is mobilized from the fiber, which massively increases the reactive potential of the iron because it comes into intimate contact with fragile biomolecules. However, upon entrance of a fiber into the lungs, two wildly different environments may be encountered, depending on the size of the fiber and the disease status of the lung. If a large fiber were to enter a healthy lung,

cellular damage may occur in the immediate vicinity of the fiber due to physical disruption of the adjacent cells by the fiber. In this case the fiber may be immediately exposed to many cellular components from normal mesothelial or endothelial cells, and a variety of signals would be sent initiating the inflammatory process. In a second condition, if a fiber were to enter a lung where inflammation had already occurred, perhaps from previous fiber exposure, a newly entered fiber could be immediately engulfed by macrophages in the vicinity. It is believed that macrophages are good sources for iron, particularly hemosiderin, ferritin, and perhaps even low-molecular-weight iron. A fiber under these conditions may have iron associated with it in an entirely different manner than in the former set of conditions, as discussed in Chapter 2.

A recently inhaled fiber, or one which has had iron bound to it, is likely to be phagocytized, if the fibers are of the proper dimension. Mobilization is very likely to occur following phagocytosis because of the low pH of the phagosome. Mobilized iron is known to be reactive with all types of biomolecules and could readily damage cellular components if it were released from the phagosome into the cytoplasm or nucleus. It is possible that the mobilized Fe(II) and Fe(III) could damage the phagosome via iron-catalyzed lipid peroxidation, and then be free to leak into the cytoplasm of the cell. Transport of iron into the

nucleus is likely to occur when iron associates with proteins destined for the nucleus or with low-molecular-weight chelators which may be capable of entering the nucleus through nuclear pores. Disruption of the phagosomal membrane may allow entrance of a fiber into the normal intracellular environment, which is generally held at pH 7.0-7.5. It is possible that low-molecular-weight chelates of iron could transfer their iron to the fiber itself, as suggested by the finding that iron can be bound to fibers from complex solutions like tissue culture media. If the cell were to die, another cell could phagocytize the fiber and re-enrich the fiber with iron, and the process of iron binding and release could continue indefinitely. The silicate structure itself, because of its ability both to bind iron or release iron depending on the environment, functions as what one might term an "iron hotel."

The work presented here has demonstrated another line of evidence that biochemically dangerous fibers are not chemically inert, but rather are highly interactive with their environment. Six varieties of fibers were examined in the work presented in this thesis: native, soaked, and DF crocidolite, as well as silicon carbide whiskers #1, #2, and #3. All of these fibers appeared to have some degree of biological activity, and all of the fibers were capable of acquiring some amount of iron from an *in vitro* inorganic iron source, namely $FeCl_2$ solution. Crocidolite and DF

crocidolite were also capable of acquiring iron from complex solutions, such as tissue culture media. This observation suggests that, in an aqueous environment like that of the cell or extracellular space, under the appropriate conditions, inhaled durable fibers may be able to bind biochemically reactive iron, which could potentiate the dangerous nature of the fiber. Additional iron bound to the fiber increases the ability of the fiber to catalyze the formation of DNA single-strand breaks *in vitro,* suggesting that additional iron on the surface of a mineral fiber may lead to enhanced biochemical reactivity of the fiber toward a number of biological targets. The ability of the crocidolite and DF crocidolite to bind iron from $FeCl_2$ solution correlated with the cytotoxic capabilities of the fibers in Syrian hamster embryo cells. The ability of crocidolite treated with various tissue culture media to form DNA SSBs *in vitro* showed a very strong correlation to the cytotoxicity of crocidolite to A549 cells cultured in the same media. These correlations lend biological significance to the work presented here. This correlation between cytotoxicity and the ability of fibers to bind iron may suggest a scheme by which the potential hazard of both man-made (i.e. silicon carbide whiskers) and naturally occurring mineral fibers to biological systems may be assessed. The potential utility of this scheme is corroborated by previous work performed in the laboratory

which found native erionite, known to be even more carcinogenic than crocidolite, to be completely void of biochemical activity as measured by the DNA SSB assay. Following binding of even catalytic amounts of iron to the erionite, however, an increase in the ability of the erionite to form DNA SSBs was observed, rendering the fiber at least as capable of damaging DNA as crocidolite. Verification of this correlation requires more extensive studies on the biological activity of newer fibers such as silicon carbide whiskers. Also, the conditions for both binding of iron and mobilization of intrinsic or acquired iron are not known, but may be critical to our understanding of the interaction of fibrous carcinogens with biological molecules and merit further investigation.

APPENDIX

July 12, 1994

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Dear Jeanne,

I hereby agree that you incorporate the paper entitled "Influence of Iron in Tissue Culture Media on Crocidolite-dependent Cytotoxicity and DNA Single-Strand Breaks" into your Masters Thesis.

Sincerely,

Chien-Chung Chao